DEFINITION OF A NEAR REAL TIME MICROBIOLOGICAL MONITOR FOR SPACE VEHICLES

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FINAL REPORT

ORIGINAL CONTAINS
COLOR ILLUSTRATIONS

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1.0 INTRODUCTION

The monitoring and evaluation of microbiological parameters onboard Freedom and other long duration missions will be essential to the health of flight personnel and to the efficient and continual operation of ECLSS systems and subsystems. Even though the technology is available to accomplish the required tasks, much development of methodology and modifications of appropriate hardware to proposed Space Station operation and conditions are required. There are well defined methods employed on Earth designed to assess water and air quality with respect to microbiological standards. Some of these methods, such as membrane filtration and multiple tube fermentation techniques for the microbiological analysis of water, could be employed on space vehicles with modification and miniaturization.

There is an inherent disadvantage in employing classical microbiological methodology in water and air analysis since all of these techniques require some time period for incubation during which the organisms present can grow. This incubation period thus dictates a lag time from the time of sampling to the time when results become available. This problem has been addressed somewhat on Earth with the development of rapid identification systems. These systems have been particularly useful in the identification of organisms from clinical samples where the rapid results is an obvious benefit. However, even with these rapid methods, a large number of cells are required.

With the employment of similar but modified methods on the longer duration missions there will be a period of uncertainty regarding the quality of water and air supplies with respect to

microbiological content. If a microbiological problem should exist at the time of sampling, in all likelihood, it will have become worse by the time the results are available. Similarly, unless rapid methods are employed there is no assurance that the microbiological quality will be preserved until the results are available. Therefore, it is desirable to decrease this period of uncertainty to a minimal amount of time.

In the past two decades many advances have been made in the detection and identification of microorganisms. For the most part, these advances are oriented toward clinical rather than environmental aspects of microbiology. Based on the information obtained to date the lowest sensitivity reported for detection of microorganisms is approximately $1x10^5$ CFU's per 100 ml. Of course, this value is considerably higher than the present specifications established for Space Station Freedom. Although technology is not currently available for the rapid detection of microorganisms at this level, many micro-chemical techniques exist which, with proper modifications, might prove to be a rapid mechanism for the detection of microorganisms aboard the Freedom Station as well as future planetary bases and longer duration missions.

2.0 IDENTIFICATION OF POTENTIAL METHODOLOGIES

2.1 Literature Review

An extensive literature review has been completed. Over 600 articles have been identified and organized by methodology. Results of the literature review is included in this report as Appendix A. The following sources were used as general references to compile and identify pertinent research articles:

Manual of Methods for General Bacteriology. Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. ASM Press. 1981.

The Viable Count. Tsutomu Hattori. Springer Verlag, New York. 1988.

Rapid Methods and Automation in Microbiology and Immunology. Habermehl, K. O., ed. Springer-Verlag, New York. 1984.

Instrumental Methods for Rapid Microbiological Analysis. Nelson, W. H., ed. VCH Publishers, Inc. 1985.

Membrane Filtration: A Users Guide and Reference Manual. Brock, T. D. Science Tech Publishers, Inc. Madison, WI. 1983.

Bioluminescence and Chemiluminescence Instruments and Applications. Van Dyke, K., ed. CRC Press. Boca Raton, FL, 1985.

The direct Epifluorescent Filter Technique for the Rapid Enumeration of Microorganisms. Pettipher, G.L., Research Studies Press, Ltd. Letchworth, HertFordshire, England, 1983.

In addition, the following computerized searches were conducted to obtain pertinent articles not referenced in the sources listed above.

Biological Abstracts
Chemical Abstracts
Engineering Index Monthly, 1987-1988.
Medline, 1983-1988

Keywords used in the computerized searches included:

Detector/Detection/Detect
Lasers
Bacteria/viruses
Epifluorescence
Biological Monitoring/Ecological Monitoring/
Environmental Monitoring
Microbiology/Air Microbiology/
Environmental Microbiology/Water Microbiology
Microbiology
Drinking water quality
Bacteriological monitoring
Microorganisms
Sterility assurance levels
Sterility maintenance

2.2 Equipment and Instrumentation

A number of "off the shelf" bacterial detection and identification systems have been identified as well. For the most part, "off the shelf" detection equipment currently available rely on either light scattering technology or electrical impedence. Three exceptions currently identified are 1) the use fluorescent techniques including microscopy and flow cytometry, 2) the use of mass spectroscopy and 3) the use of hydrogen ion concentration detected electrochemically. Many of the systems available are sensitive only at levels of approximately 10^3 cells per milliliter or higher and therefore require a growth step for detection. Manufacturers of microbiological detection and monitoring systems are presented in Appendix B.

Microbiological identification systems currently available include standard and automated biochemical profiling, serological testing and fatty acid profiling. Manufacturers of identification systems are just as numerous as those listed for detection and monitoring but most are devoted to clinically

important microorganisms, require the use of pure cultures and require relatively large numbers of cells. Manufacturers of identification systems are also listed in Appendix B.

2.3 Potential Methodologies

A number of potential methods have been identified which have the capability to detect microorganisms. A list of the methodologies identified, researched and included for evaluation is presented in Table 1. A brief description of each methodology and additional pertinent information which could be identified has been included for review (Appendix A).

Table 2.1

Identified Methodologies

1. Viable Plate Count/Membrane filter technique
2. Viable Plate Count/Spread or Pour-plate
3. Direct viable count
4. Laser light scattering
5. Primary fluorescence
6. Flow cytometry with fluorochrome detection
7. Direct epifluorescent filter technique (AODC)
8. Immunofluorescence
9. Electrical Impedence/The Bactometer
10. Electrical Impedence/The Coulter Counter
11. Scanning Electron Microscopy
12. Limulus Ameobocyte Lysate Assay
13. Luciferase
14. Specific Enzyme Assays
15. Fatty acid gas chromatography
16. Raman Spectroscopy
17. FTIR Spectroscopy
18. GC/Mass Spectometry
19. MS/MS
20. Visible Spectroscopy
21. UV Spectroscopy
22. Pyrolysis/Gas Chromatography
23. Radioimmunoassay (RIA)
24. Volatile Product Detection/Membrane Filtration
25. Thermal Photometry
26. Chemiluminescence
27. Microcalorimetry
28. Polymerase Chain Reaction
29. Surface Acoustic Wave Detector

3.0 EVALUATION AND TRADES

3.1 Evaluation Scheme

After much consideration, the evaluation scheme illustrated by Figure 1 was devised. All identified methodologies were subjected to the primary evaluation criteria. These criteria, which include sensitivity, analysis time, technological maturity and broadness of application, were identified as most important for a microbiological monitor. In order to progress to the next evaluation phase the proposed methodology had to obtain a minimum of 75% of the total points available. All methodologies which received less than 75% of the total points available from the primary technical evaluations were eliminated from further investigation.

Methods which received 75% or greater were then evaluated using the Secondary Evaluation Criteria. The secondary technical criteria included sampling, post sampling, analysis, hardware and post analysis considerations. Following this evaluation the six highest scoring methodologies continued through the evaluation/trade scheme.

Next, engineering and feasibility evaluations were performed on each of the six candidate methods. From this information, two or three methodologies were to be identified as candidate microbiological monitors to be evaluated during Phase II. However, as discussed later in Section 4.0, five candidate monitors have been identified which will be evaluated further in the Phase II efforts. The evaluation criteria defined and used to date are presented in Section 3.2 of this report.

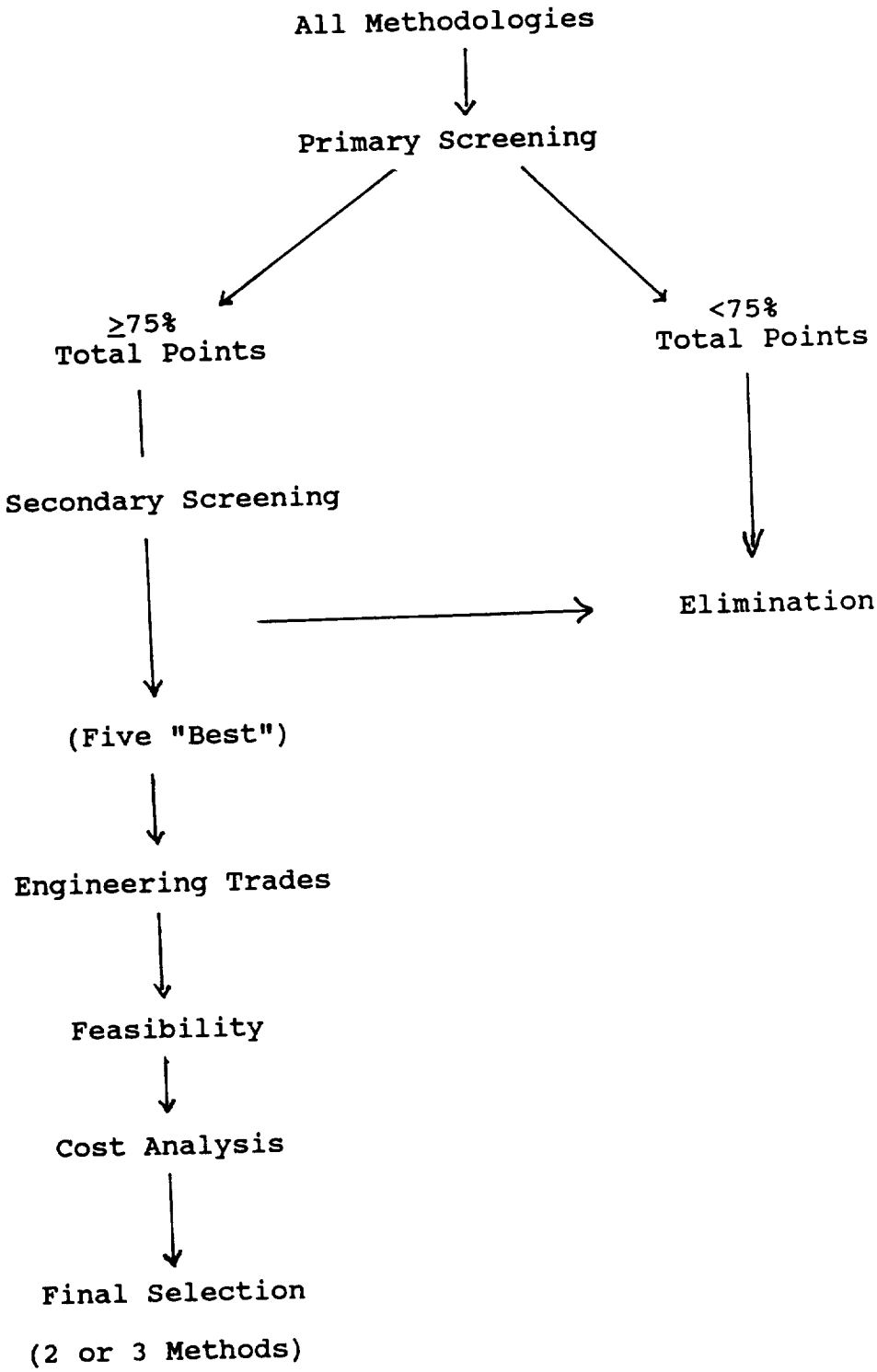

Figure 1. Evaluation/Trade Scheme

3.2 Evaluation Criteria

The criteria used to evaluate the candidate methodologies are divided into three categories: 1) Technical, 2) Engineering and 3) Feasibility. Each of these categories are discussed below.

3.2.1 Technical Evaluation Criteria

The technical evaluation criteria are subdivided into two categories, primary and secondary. Criteria which were used in the technical evaluations are discussed below.

3.2.1.1 Primary Evaluation Criteria

The primary evaluation criteria are those criteria which, in our opiniun, must be met for a useful and reliable microbiological monitor. They include sensitivity, time requirements, technological maturity and the broadness of application. Each of these primary criteria are judged to be of equal importance and have been given a weighting of five (5) points.

Sensitivity: With the specifications for potable water currently set at less than one colony forming unit per 100 ml, it is obvious that a highly sensitive method is called for. Using this scheme sensitivity can be evaluated at different levels of acceptable detection. Because of the current specifications only 1CFU/100mL will be presented. The following rating scheme was used for this criterion:

The method is capable of single cell detection per 100 mL:

	Rating
Always satisfied	4
Most often satisfied	3
Acceptable	2
Marginally acceptable	1
Not Acceptable	0

Rapidity of obtaining results: The more rapid results can be obtained, the less storage time and containment time of the processed water is required. A real time monitor would allow for monitoring at the point of use and thus is given the highest rating. The following rating scheme was used for this criterion:

	Rating
Real-time results less than 30 min	4
Near Real-time results 30 min to 6 hrs	3
Moderately rapid 6 to 18 hrs	2
Traditional 18 to 48 hrs	1
Exceeds 48 hrs	0

Technological Maturity: In order for the monitor to be applicable for use on Space Station Freedom the technological maturity of the method and subsequent required equipment are extremely important. The ideal associated hardware would be off the shelf equipment requiring little or no modification. The following rating scheme was used for this criterion:

	Rating
Off Shelf Equipment (no modification)	4
Off Shelf Equipment (minor modifications)	3
Off Shelf Equipment (moderate modifications)	2
Off Shelf Equipment (extensive modification)	1
Off Shelf Equipment Not Available	0

Broadness of Application: The ideal microbiological monitor would be capable of detecting all major groups of microorganisms including bacteria, fungi, protozoa and viruses. A technology which has the capability to detect all four groups would be evaluated highest. The following rating scheme was used for this criterion:

	Rating
All Major Groups	4
Three Major Groups	3
Two Major Groups	2
One Major Group	1
No Complete Group	0

3.2.1.2 Secondary Evaluation Criteria

A total of thirteen (13) secondary technical evaluation criteria were identified. These criteria are sub-divided into four categories which include: sampling, post-sampling/pre-analysis, analysis, and post analysis considerations. A description and justification of each of the criteria, as well as the weighting factors used for the secondary evaluation is presented below:

3.2.1.2.1 Sampling Considerations

The more simple the sampling procedure required the more desirable the method. This criteria is is divided into three categories which further serves to describe sampling simplicity. Each of these are presented below:

No physical removal of the sample is required: Procedures requiring the physical removal of a sample prior to analysis increase the complexity of the monitoring system and also pose potential avenues for containment breaches. A weighting of (5) is given to this criterion. The following rating scheme was used for this criterion:

	Rating
No physical sample removal required	4
Sample removal is required	0

Sampling technique requires crew involvement: The requirement for crew involvement for sampling is undesirable. The recommended weighting for this criterion is (3) since it is of less importance than the sample removal requirement. The following rating scheme was used for this criterion:

	Rating
No crew involvement required	4
Sampling technique requires simple flip of switch (automated)	3
Sampling technique requires minimal crew efforts to obtaining the sample	2
Sampling technique requires moderate crew effort to obtain the sample	1
Sampling technique is crew intensive	0

Non-invasive: A method requiring an invasive procedure could present some problem regarding maintenance of process stream containment. Thus, an invasive technique is considered undesirable and a weighting of 5 is recommended. The following rating scheme was used for this criterion:

	Rating
Procedure is non-invasive	4
Procedure is invasive	0

3.2.1.2.2 Post Sampling Considerations

The amount of post sampling activity required to actually process the sample is of great importance. These considerations will influence the equipment that is required, the time required for analysis and in some cases crew involvement. These activities include sample concentration and pre-analysis manipulation. Each of these criteria are defined below.

Sample Concentration: In some cases post collection concentration of the sample is required to obtain the desired detection limits. In many cases this is easily accomplished but it does require additional steps over a method which does not require sample concentration to achieve the desired detection limits. The recommended weighting for this criterion is 1. The following rating scheme was used for this criterion:

	Rating
No sample concentration is required	4
Sample concentration is required	0

Manipulation: The less manipulation of the sample after sample acquisition, the more desirable the method. Post sampling manipulation could require complete crew involvement, partial crew involvement, complete robotic manipulation, or no manipulation. Since manipulation could require a significant amount of crew time this criterion is given a weighting of 4. The following rating scheme was used for this criterion:

	Rating
No post sampling manipulation	4
Robotic post sampling manipulation	3
Minimal crew manipulation required	2
Moderate crew involvement required	1
Total crew involvement required	0

3.2.1.2.3 Analysis considerations

Analysis considerations are those which impact on the actual sample analysis and results obtainable. Important considerations identified are the potential for confirming analysis, subsequent characterizations (identification and antibiotic sensitivity) and discriminatory capability. These criteria are defined below.

Non-destructive method: A destructive method of monitoring would not allow for further analysis, identification, determination of antibiotic sensitivities and archiving of the organisms encountered in the process stream. On the other hand, a destructive mechanism would allow for elimination of detected microorganisms. This is given a weighting of 4. The following rating scheme was used for this criterion. The order for evaluation can be reversed should destruction of the detected microorganism prove to be desirable.

	Rating
Method retains viability ----------	4
Method kills ----------	0

Identification potential: The method provides information regarding the identification of the microorganism detected. This criterion is given a weighting of 2. The following rating scheme was used for this criterion:

Identification Level		Rating
Species	-------	4
Genus	-------	3
Sub-groups (Gram positive bacteria, Gram negative bacteria, etc)	-------	2
Major group (bacteria, fungi, protozoa)	-------	1
No information obtainable	-------	0

Discriminatory Capability: The method has the capacity to discriminate between living microorganisms and inanimate particulate contaminants of similar sizes. The recommended weighting for this criterion is 4. The following rating scheme was used for this criterion:

	Rating
Always satisfied	4
Most often satisfied	3
Acceptable	2
Marginally acceptable	1
Not Acceptable	0

3.2.1.2.4 Hardware Considerations

Because of the relative importance of unnecessary duplication of monitoring and analysis equipment and the benefits provided by methods utilizing similar equipment it was felt that hardware considerations must be evaluated. This would include the integration with other instruments to yield additional information regarding microbial contamination and the capacity for hardware to perform multiple tasks, i.e. chemical and biological monitoring. At the present time, neither of these considerations are heavily weighted. In addition, hardware and integration criteria may be further evaluated during the engineering trades. These criteria are defined below.

Integration capability with other methods: The ability to interface two or more methods would increase the amount of information regarding any detected microorganism and would add to identification potential. This criterion is given a weighting of 1. The following rating scheme was used for this criterion:

	Rating
Readily integrated into a more complex system	4
Not easily integrated into a more complex system	0

Integration capability with the chemical monitor: Instrumentation that could be used for both chemical and microbiological monitoring is desirable since this would decrease the overall load required for the monitoring systems. This criterion is given a weighting of 1. The following rating scheme was used for this criterion:

	Rating
Method employs instrumentation that is applicable to both chemical and microbiological monitoring	4
Method is restricted to microbiological monitoring	0

3.2.1.2.5 Post analysis considerations

At the present time, post analysis considerations consist primarily of those criteria which relate to the by-products of the given methodology. These would include the production of expendable waste products, as well as chemical and biological hazards to the environment and crew. These criteria are defined below.

Generation of waste mass: Any method that generates a material that must be stored, processed, or shipped back to earth is, to some degree, undesirable. This criterion is given a weighting of 3. The following rating scheme was used for this criterion:

	Rating
No waste mass generated	4
Minimal waste mass generated	3
Moderate waste mass generated	2
Moderate to High waste mass	1
Extensive waste mass generated	0

Biohazard generation: Material designated as biohazardous include any waste that contains potentially infectious material, such as viruses, bacteria, fungi, or protozoa. Biohazard generation is undesirable. Any biohazardous waste would require special processing or containment. This criterion is given a weighting of 3. The following rating scheme was used for this criterion:

	Rating
No biohazard generation	4
Minimal biohazardous waste	3
Low levels of biohazardous waste	2
Moderate levels of biohazardous waste	1
High levels of biohazardous waste	0

Chemical hazard generation: Waste in this category would include all chemicals known to be irritating, toxic, mutagenic, or carcinogenic. This type of waste generation is undesirable. Any chemical waste would require special processing or containment. This criterion is given a weighting of 3. The following rating scheme was used for this criterion:

	Rating
No chemical hazard generation	4
Minimal levels of chemical hazard	3
Low levels of chemical hazard	2
Moderate levels of chemical hazard	1
High levels of chemical hazard	0

3.2.2 Engineering Criteria

The following is a discussion of the engineering criteria which were established as important to the definition and development of a microbiological monitor for space applications.

Power: Power useage requirements are extremely important to the definition and development of a microbiological monitoring device for use in the Space environment. Power constraints are generally prohibitive for equipment and instrumentation having large power requirements. This criterion is given a weighting of five (5). The following rating scheme was used for this criterion:

	Rating
Less than or equal to 0.5 Kw	4
Less than or equal to 1.0 Kw	3
Less than or equal to 1.5 Kw	2
Less than or equal to 2.0 Kw	1
Greater than 2 Kw	0

Weight Requirements: Because of limited launch weight constraints hardware weight requirements are critical. Equipment and hardware having excessive weight which cannot be effectively reduced by miniaturization is prohibitive. This criterion was given a weighting of five (5). The following rating scheme was used for this criterion:

	Rating
Equipment and all accessory components weigh less than 25 lbs	4
Equipment and all accessory components weigh less than 50 lbs	3
Equipment and all accessory components weigh less than 75 lbs	2
Equipment and all accessory components weigh less than 100 lbs	1
Equipment and all accessory components weigh more than 100 lbs	0

Volume Requirements: The volume or space requirements for a candidate system are equally important in Space Applications where space is limited. The smaller the equipment or instrumentation required to perform the task the better. Equipment and instrumentation that is large or has numerous components which cannot be miniaturized is prohibitive. This criterion was given a weighting of five (5). The following rating scheme was used for this criterion:

	Rating
Equipment can be miniaturized to fit a single rack	4
Equipment can be miniaturized to fit a double rack	2
Equipment cannot be miniaturized to fit a double rack	0

Expendable Storage Requirements: In addition, to the volume requirements of the hardware itself certain expendibles may be required for continuous operation. With a resupply schedule of approximately thirty (30) days this volume requirement could be substantial. This criterion was given a weighting of five (5). The following rating scheme was used for this criterion:

	Rating
Expendible storage can be limited to a single rack	4
Expendible storage can be limited to a double rack	2
Expendible storage requires more than a double rack	0

Waste Storage Requirements: Some candidate methods will produce waste material which has to be stored between STS visits. Obviously, methods and procedures which produce no waste and therefore have no waste storage requirements are ideal. In addition, a waste storage requirement may have safety limitations as well. This criterion was given a weighting of five (5). The following rating scheme was used for this criterion:

	Rating
Method requires no waste storage	3
Method requires minimal waste storage	2
Method requires moderate waste storage	1
Method requires excessive waste storage	0

3.2.3 Feasibility Criteria

Feasibility criteria were established in order to rate the methodologies based upon the feasibility of actually developing the concept, methodology as well as to modify and build the required hardware to perform the monitoring task under microgravity conditions. Even if the technical and engineering evaluations score high if the feasibility is low there is a good chance of failure during the development phase or under flight conditions. The "proof of concept" of each candidate methodology has been previously demonstrated. For this reason, it was not felt that proof of concept was a useful criteria for this evaluation. Normally, one might include accuracy and precision of the candidate methodology as part of the feasibility evaluation, however neither of these parameters can be demonstrated for all methods at this time. The following criteria have been defined as important to the feasibility of individual candidate development and in-flight operation. All feasibility criteria have been assigned a weighting of five (5).

Validity of Measurement: The validity of the measurement may be defined as the ability of the candidate instrument and associated method to measure what it is suppose to, in this case microorganisms. In other words, can the candidate methodology detect microorganisms from background interferences. The following rating scheme was used for this criterion:

	Rating
Validity is acceptable	4
Validity is questionable	2
Validity is unacceptable	0

System Complexity: The complexity of the candidate system is important to both the feasibility of development and operation. If the system is too complex it will be dificult to develop, build and operate. In addition, the complexity of the candidate system will be directly related to the ability of the crew to troubleshoot and repair problems in-flight. The following rating scheme was used for this criterion:

	Rating
System is simple with few components	4
System is moderately complex or has several components	2
System is complex or has many components	0

System Maintenance Requirements: Excessive system maintenance either ground-based or in-flight is a major disadvantage. Candidate systems which have frequent or major maintenance requirements are undesirable for in-flight systems. The following rating scheme was used for this criterion:

	Rating
System has low maintenance requirements	4
System has moderate maintenance requirements	2
System has excessive maintenance requirements	0

Microgravity Compatibility: Compatibility in microgravity and\or reduced gravity environments is of upmost importance since this is the ultimate environment for which the system is to be used. The following rating scheme was used for this criterion:

	Rating
Little problem anticipated for microgravity application	4
Some problem anticipated but not insurmountable	2
Major problems anticipated for microgravity application	0

In-flight Calibration: Calibration of the candidate system will be important to the verification of system performance as well as to quantify contamination levels. The capability of in-flight calibration will be important to long duration activities and missions. This criteria is view as extremely important to the application aboard Freedom, on Lunar bases and the during the planned Mars mission. The following rating scheme was used for this criterion:

	Rating
In-flight calibration possible	4
In-flight calibration not possible	0

3.2.4 Cost

Cost estimates regarding both the development of hardware and methodology for the application of each final candidate technology to microbiological monitoring were not considered in the evaluations. It should be noted that cost estimates for development of each candidate monitor will be included in the Phase II proposal. It is the opiniun of the authors that cost should not be a factor when the health maintenance and safety of the crew are concerned.

4.0 EVALUATION RESULTS

A number of methods applicable for the detection and enumeration of microorganisms have been defined. Each of these have appropriate applications for various types of monitoring. The major issues at hand regarding the assessment of viable microbial populations in any particular environment relate to the understanding of the advantages, disadvantages, limitations and applicability of the methods defined to date. In the final analysis, the method of choice must be dictated by the system under investigation and the data generated must be interpreted with consideration of the limitations imposed by the method used.

A Lotus program was established to rank the identified methodologies based on the specific weighting factors assigned to each criteria and the rating of each methodology within these criteria as previously defined.

4.1 Results of the Technical Evaluation

Although the criteria weighting and ratings given to each of the methodologies are somewhat subjective, great care was taken to maintain consistancy throughout the rating process. Because direct laboratory comparisons of all of the methodologies is not practical, the rating of the various technologies was based largely on data obtained from primary research publications and technical bulletins. A limited appraisal of the applicability and sensitivity of laser light scattering was performed since this method was a high ranking method and we were able to obtain access to this instrumentation for a short period of time.

Since no single method could meet all of the criteria which were established, it was felt that a fatal flaw analysis was not feasible. Rather, we applied a relative scoring analysis discriminating a total of 17 criteria. The identification, definition and weighting of each of the criteria was the result of numerous discussions among our investigators. All of the methods were rated according to the specified criteria relative to each other. In many cases, the final ratings presented are the averages obtained from the individual ratings assigned by several investigators.

The results of the Technical Evaluations are presented in Tables 4.1, 4.2, and 4.3. Table 4.1 illustrates the results of the Primary Screening. Twenty-nine methods were identified which have potential application to microbiological detection. All methods were evaluated based on the primary criteria established and discussed previously. Those methodologies obtaining 75% of the total available points were further evaluated using the secondary evaluation criteria.

Approximately one-third of the initial methodologies were evaluated using the secondary criteria. Table 4.2 illustrates the results of the Secondary screening. Also included in Table 4.2 is the Viable Plate Count/Membrane Filtration Technique for comparison purposes.

Table 4.3 summarizes the combined results obtained from the Technical Evaluation. The methods listed in Table 4.3 are ranked in order of highest to lowest. The six (6) highest scoring methods were then evaluated using the engineering and feasibility criteria. These included: laser light scattering, electronic

particle counting, primary fluorescence, surface acoustic wave detection, secondary fluorescence/flow cytometry, and volatile product detection coupled with membrane filtration.

Table 4.1

MICROBIAL MONITOR FOR SPACE VEHICLES
PRIMARY EVALUATION WORKSHEET

Criterion	Weighting (0-5)	Highest Rating Possible	Highest Score Possible	METHODS 1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18		19		20		21		22		23		24		25		26		27	
				eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW		eW	
Sensitivity																																																								
1 CFU per 100mL	5	4		3		0		4		3		3		3		4		4		2		4		4		0		3		2		2		2		2		2		2		2		2		3		4		3		2	
			20		15		0		20		15		15		15		20		20		10		20		20		0		15		10		10		10		10		10		10		10		10		15		20		15		10
10 CFU per 100mL	0	4		3		0		4		4		4		3		4		4		3		3		4		0		4		2		2		3		3		3		3		3		3		3		4		3		3	
			0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0		0	
Rapidity of obtaining results	5	4		0		0		2		4		3		3		3		3		1		4		2		3		3		2		2		2		2		2		2		1		1		2		2		3		3		2		2	
			20		0		0		10		20		15		15		15		15		5		20		10		15		15		10		10		10		10		10		10		5		5		10		10		15		15		10		10
Technological maturity	5	4		4		4		4		4		3		3		3		3		3		3		3		3		3		3		4		2		3		4		3		4		4		4		3		4		0		3		1	
			20		20		20		20		20		15		15		15		15		15		15		15		15		15		15		20		10		15		20		15		20		20		20		15		20		0		15		5
Broadness of Application	5	4		2		2		2		3		3		3		2		0		2		3		4		0		3		2		1		2		2		2		4		2		2		3		0		2		3		3		2	
			20		10		10		10		15		15		15		10		0		10		15		20		0		15		10		5		10		10		10		20		10		10		15		0		10		15		15		10
Total			80		45		30		60		70		60		60		60		50		40		70		65		30		60		45		45		40		45		50		55		45		45		55		35		60		50		55		35

Key of Potential Methodologies

1. Membrane Filter Technique
2. Spread or Pour-plate
3. Direct Viable Count
4. Laser light scattering
5. Primary fluorescence
6. Flow cytometry
7. Epifluorescent Filter Technique
8. Immunofluorescence
9. Bectometer
10. Coulter Counter
11. Scanning Electron Microscopy
12. Limulus Amoebocyte Lysate
13. Bioluminescence
14. Specific Enzyme Assays
15. Fatty acid gas chromatography
16. Raman Spectroscopy
17. FTIR Spectroscopy
18. GC/Mass Spectometry
19. MS/MS
20. Visible Spectroscopy
21. UV Spectroscopy
22. Pyrolysis/Gas Chromatography
23. Radioimmunoassay (RIA)
24. Volatile Product Detection / MF
25. Thermal Photometry
26. Chemiluminescence
27. Microcoulumitry
28. Polymerase Chain Reaction

Table 4.2

MICROBIAL MONITOR FOR SPACE VEHICLES
ECONDARY EVALUATION WORKSHEET

Criterion	Weighting (0-5)	Highest Rating Possible	Highest Score Possible	METHODS 1		3		4		5		6		7		10		11		13		24		26		29	
					*W		*W		*W		*W		*W		*W		*W		*W		*W		*W		*W		*W
o Physical emoval of Sample	5	4	20	0	0	0	0	4	20	4	20	4	20	0	0	4	20	0	0	0	0	0	0	0	0	4	20
No Crew nvolvement	3	4	12	3	9	2	6	4	12	4	12	4	12	3	9	4	12	0	0	2	6	3	9	4	12	4	12
"o Concentra Required	1	4	4	0	0	0	0	4	4	4	4	4	4	0	0	4	4	0	0	4	4	0	0	0	0	4	4
Manipulation	1	4	4	0	0	0	0	4	4	4	4	3	3	3	3	4	4	0	0	1	1	3	3	3	3	4	4
on-invasive	5	4	20	0	0	0	0	4	20	4	20	0	0	0	0	4	20	0	0	0	0	0	0	0	0	0	0
on-destruct	4	4	16	4	16	0	0	4	16	0	0	0	0	0	0	4	16	0	0	0	0	4	16	0	0	4	16
ID potential	2	4	8	2	4	1	2	1	2	3	6	2	4	1	2	0	0	1	2	0	0	3	6	0	0	2	4
eneration o waste mass	3	4	12	1	3	1	3	4	12	4	12	3	9	2	6	4	12	2	6	2	6	1	3	2	6	3	9
iohazard eneration	3	4	12	0	0	3	9	4	12	4	12	4	12	4	12	4	12	4	12	4	12	4	12	0	0	4	12
hemical haz eneration	3	4	12	3	9	3	9	4	12	4	12	2	6	1	3	4	12	1	3	3	9	3	9	2	6	4	12
Integration apability with ther methods	1	4	4	4	4	4	4	4	4	4	4	4	4	4	4	0	0	4	4	0	0	0	0	4	4	0	0
ntegration apability with chemical monitor	1	4	4	0	0	0	0	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0	0
iscriminato Capability	4	4	16	4	16	4	16	0	0	1	4	2	8	2	8	0	0	3	12	3	12	4	16	2	8	4	16
Total			144		61		49		122		114		82		43		116		35		50		70		47		111

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TABLE 4.3

SUMMARY OF THE TECHNICAL EVALUATION

Method Number	Description	Technical		Total Points
		Primary	Secondary	
0	Maximum Available Points	80	144	224
4	Laser Light Scattering	70	122	192
10	Electronic Particle Counting	70	116	186
5	Primary Fluorescence	60	114	174
29	Surface Acoustic Wave Detector	60	111	171
6	Secondary Fluoresence	60	82	142
24	Volatile Product/MF	60	76	136
13	Bioluminescence	60	54	114
3	Direct Viable Count	60	53	113
1	Membrane Filtration	45	67	112
26	Chemiluminescence	55	55	110
7	Epifluorescence	60	49	109
11	Electron Microscopy	65	35	100

4.2 Results of the Engineering Evaluation

The six candidate methodologies having the highest scores from the Technical Evaluation were further evaluated using the engineering criteria previously defined (Section 3.2.2). Three of the methods, laser light scattering, primary fluorescence and surface acoustic wave detection, scored quite high (≥ 85/95 total points) using these criteria (Table 4.4). The secondary fluorescent method received 75/95 total points, while electronic particle detection and volatile product detection received 65/95 and 45/95 total points, respectively.

4.3 Results of the Feasibility Evaluation

Lastly, each of the candidate methodologies were evaluated using the feasibility criteria previously described (Section 3.2.3). The results of this evaluation is presented in Table 4.5. As indicated, the secondary fluorescent method rated the maximum number of possible points. The surface acoustic wave detection method ranked second with 90/100 possible points. Laser light scattering, primary fluorescence and volatile product detection each scored 80/100 possible points. Electronic particle detection ranked last in the Feasibility Evaluation with 40/100 possible points.

Table 4.4

MICROBIAL MONITOR FOR SPACE VEHICLES
ENGINEERING EVALUATION WORKSHEET

Criterion	Weighting	Highest Rating Possible	Highest Score Possible	METHODS 4	*W	5	*W	6	*W	10	*W	24	*W	29	*W
Power Requirements	5	4	20	4	20	4	20	4	20	4	20	1	5	4	20
Weight Requirements	5	4	20	2	10	2	10	2	10	0	0	1	5	3	15
Volume Requirements	5	4	20	4	20	4	20	4	20	2	10	2	10	4	20
Expendible Storage Requirements	5	4	20	4	20	4	20	4	20	4	20	4	20	4	20
Waste Storage Requirements	5	3	15	3	15	3	15	1	5	3	15	1	5	3	15
Total			95		85		85		75		65		45		90

Table 4.5

MICROBIAL MONITOR FOR SPACE VEHICLES
ASIBILITY EVALUATION WORKSHEET

Criterion	Weighting	Highest Rating Possible	Highest Score Possible	METHODS 4	*W	5	*W	6	*W	10	*W	24	*W	29	*W
lidity of Measurement	5	4	20	2	10	2	10	4	20	2	10	4	20	2	10
stem Complexity	5	4	20	4	20	4	20	4	20	0	0	2	10	4	20
stem Maintenance Requirements	5	4	20	4	20	4	20	4	20	0	0	2	10	4	20
Microgravity Compatibility	5	4	20	2	10	2	10	4	20	0	0	4	20	4	20
-flight Calibration	5	4	20	4	20	4	20	4	20	4	20	4	20	4	20
Total			100		80		80		100		30		80		90

4.4 Summary

The technical evaluations were established to identify pertinent minimum criteria required for a near real-time microbiological monitor. In addition, this allowed for the screening of a large number of potentially applicable techniques and instrumentation against a large number of identified technically important criteria. Each of the candidate methodologies met or exceeded the minimum requirements set forth in the technical evaluation. The candidate methodologies therefore, represented the "best" techniques currently available based on the identified criteria and the weighting factors assigned to each. The purpose of the engineering evaluation was to compare the candidate methodologies based on specific design limitations in order to identify weak or problem areas associated with the development and deployment of candidate hardware for use in Space applications. The feasibility evaluation, on the other hand, was incorporated in order to compare the candidate methodologies based on the actual feasibility of developing the technology and modifying current instrumentation to meet the requirements and specifications for a near real-time microbiological monitor in Space applications.

Table 4.6 illustrates the comparative ranking of the candidate methodologies based on the collective results from the engineering and feasibility evaluations. As illustrated by Table 4.6 there is a clear separation between the candidate methodologies based on these evaluation criteria.

TABLE 4.6

SUMMARY OF THE ENGINEERING AND FEASIBILITY EVALUATIONS

Method Number	Description	Engineering	Feasibility	Total Points
6	Secondary Fluorescence	75	100	175
4	Laser Light Scattering	85	80	165
5	Primary Fluorescence	85	80	165
29	Surface Acoustic Wave Detector	80	85	165
24	Volatile Product Detection	45	80	125
10	Electronic Particle Detection	65	30	95

*Maximum available points: Engineering 95; Feasibility 100

As illustrated, many different technologies can be identified which are applicable to microbiological monitoring. However, only a handful of these can satisfactorily meet the stringent technical, engineering and feasibility requirements for Space application. In fact, no single method currently available can meet all criteria established for a real-time microbiological monitor. Trade-offs in sensitivity, sampling, etc., are usually made at the expense of discriminatory capability, identification potential and non-destructive analysis techniques.

4.5 Recommendations

The sampling, monitoring and evaluation of microbiological parameters in space vehicles presents several unique problems. The first of these concerns the fact that the sample matrix of each is extremely different, e.g. gas, liquid, solid. On the

surface this suggests multiple methods for sample collection and handling may be required. A desirable alternative is to develop a method for microbiological monitoring that is applicable to all sample matrices of interest. With respect to the defined areas where microbiological content is a concern, all samples could be easily transferred to a liquid medium. Since liquids are conveniently analyzed by conventional microbiological methodologies it is logical that the basic technology regarding microbial analysis for Space application should be designed toward liquid samples.

Based on the engineering and feasibility evaluations it is the recommendation of this study that the candidate monitors for Phase II evaluation include as a minimum secondary fluorescence, primary fluorescence, surface acoustic wave detection, and laser light scattering. Unfortunantly, only two of the six candidate methodologies (primary fluorescence and volatile product detection) have a potential for providing information regarding the identification of the contaminant. Of these, only volatile product detection has been routinely used as an identification method.

Originally, it was our hope that the number of technologies to be evaluated during Phase II testing would be limited to two or three. However, since no single method or instrumental procedure can meet all of the desirable criteria, it is our belief that exclusion of any methodology having a high potential for development, at this time, is not prudent. For this reason, with the exception of electronic particle detection, all of the

six candidate methodologies are recommended to proceed to Phase II bench evaluations. Since primary (native) fluorescence and secondary (aided) fluorescence possess identical instrumentation requirements additional instrumentation is not required for evaluation of both. The subsequent Phase II proposal and associated test plan will reflect the above recommendations.

5.0 DESCRIPTION OF CANDIDATE MONITORS

The specific procedures and instrumentation identified in this section should not viewed as the final methodology which may be used. They are simply presented as a general outline of how a particular technique or instrument may be used. The optimal operating procedures and configurations will be defined during the comparative bench tests conducted during Phase II. A brief description of the five (5) candidate monitors are presented below. An engineering diagram of each will be included in the Phase II proposal.

5.1 Laser Light Scattering

Laser light scattering is currently a "state of the art" technology for detection and characterization of particulate contamination. It can be used for both liquid and gaseous samples. The techniques employed using this methodology allow for a non-invasive monitoring of air and water. Generally, lasers are interchangeable depending on the requirements for analysis and the parameters of interest. Figure 5.1 illustrates a candidate system for microbial monitoring.

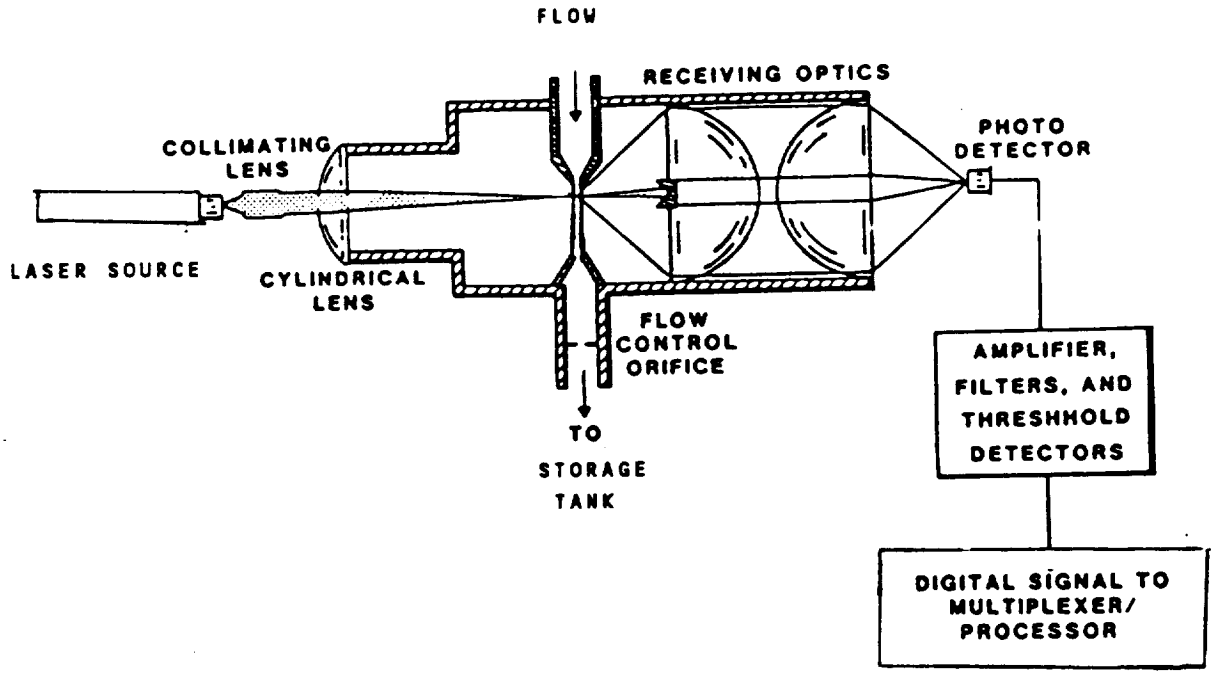
During the contract period a visit was made to Wyatt Technologies, Inc. located in Santa Barbara, CA to evaluate this methodology and instrumentation. The limited studies performed during this visit indicated that laser light scattering appears to be capable of achieving the required detection limits and does represent a relatively simple technology and instrumentation which is advantageous for space applications. The problem that became most obvious from these studies is that any particle in

the "target" size range is detected and counted. Under the conditions imposed for this "off site" evaluation it was not possible to produce a water product (diluent) that was free from particulates and thus maximize the sensitivity over the background. Thus, the major drawback is the lack of discriminatory capacity between inanimate particles and bacterial cells. However, this particular instrument has the advantage over competitive instruments in that fifteen separate scattering angles are monitored simultaneously (Table 5.1). Software is currently available for the control and operation of the instrument and to allow time-evaluation of single particles and their individual light scattering properties. With further characterizations of bacterial samples with the new software, it might be possible to differentiate between microbes and inanimate particles. Simple modifications to this technology would allow for spectrophotometric properties to be evaluated which would increase the discriminatory capabilities of this technique. In addition, the rapid response and large number of repetitive measurements which can be achieved in a short period of time should positively affect precision and accuracy. Using this procedure total volume analysis is possible thus eliminating any sample size/distribution problems associated with an invasive sampling technique.

Table 5.1

Relative Position of Detectors in Wyatt Technology Instrument

Detector	Angle from Light Source
1	23.07
2	28.96
3	34.92
4	40.97
5	47.16
6	53.49
7	60.00
8	66.73
9	73.74
10	81.08
11	88.85
12	97.18
13	106.26
14	116.42
15	128.32



Liquidborne Particle Detector

Figure 5.1

5.2 Primary (native) Fluorescence

In some molecules, absorption of light radiation produces light emission at a longer wavelength. This phenomenon is a form of luminescence known as fluorescence or phosphorescence. Fluorescence differs from phosphorescence mainly in emission persistence with fluorescence having a much shorter decay after a pulse of light. Fluorescence decay times are measured in nanoseconds (1-100 nanoseconds) while phosphorescence lifetimes may last as long as 100 seconds. Since phosphorescence requires special environmental preparations, it does not lend itself to real-time, on-line measurement.

A large number of molecules present in cells have fluorescent properties and could serve as "detection markers". Some of these molecules and their excitation and emission wavelengths are presented in Table 5.2.

Of these molecules, NADH/NADPH is recommended as the bacteria marker of choice for the following reasons:

1. All living cells contain NADH/NADPH.
2. The peak excitation wavelength in the 340nm region is consistent with the use of a nitrogen laser as an excitation light source.
3. The pulsed nature of the nitrogen laser with its high peak powers (4 kilowatts) provides a potential for high sensitivity.
4. At the same time, the low average power (millowatts) minimizes energy usage on a space vehicle and allows for a compact air-cooled design.
5. Nitrogen lasers are currently available at reasonable cost in compact form and are highly reliable in operation.
6. The short pulse width of the nitrogen laser allows for the use of time-resolved fluorescence analysis to identify as well as detect all known bacteria.

7. The use of NADH/NADPH as a bacteria detector and cell concentration marker has already been proven in industrial applications.

8. The use of a nitrogen-laser based time-resolved fluorometer for bacteria detection and identification has been demonstrated in the laboratory.

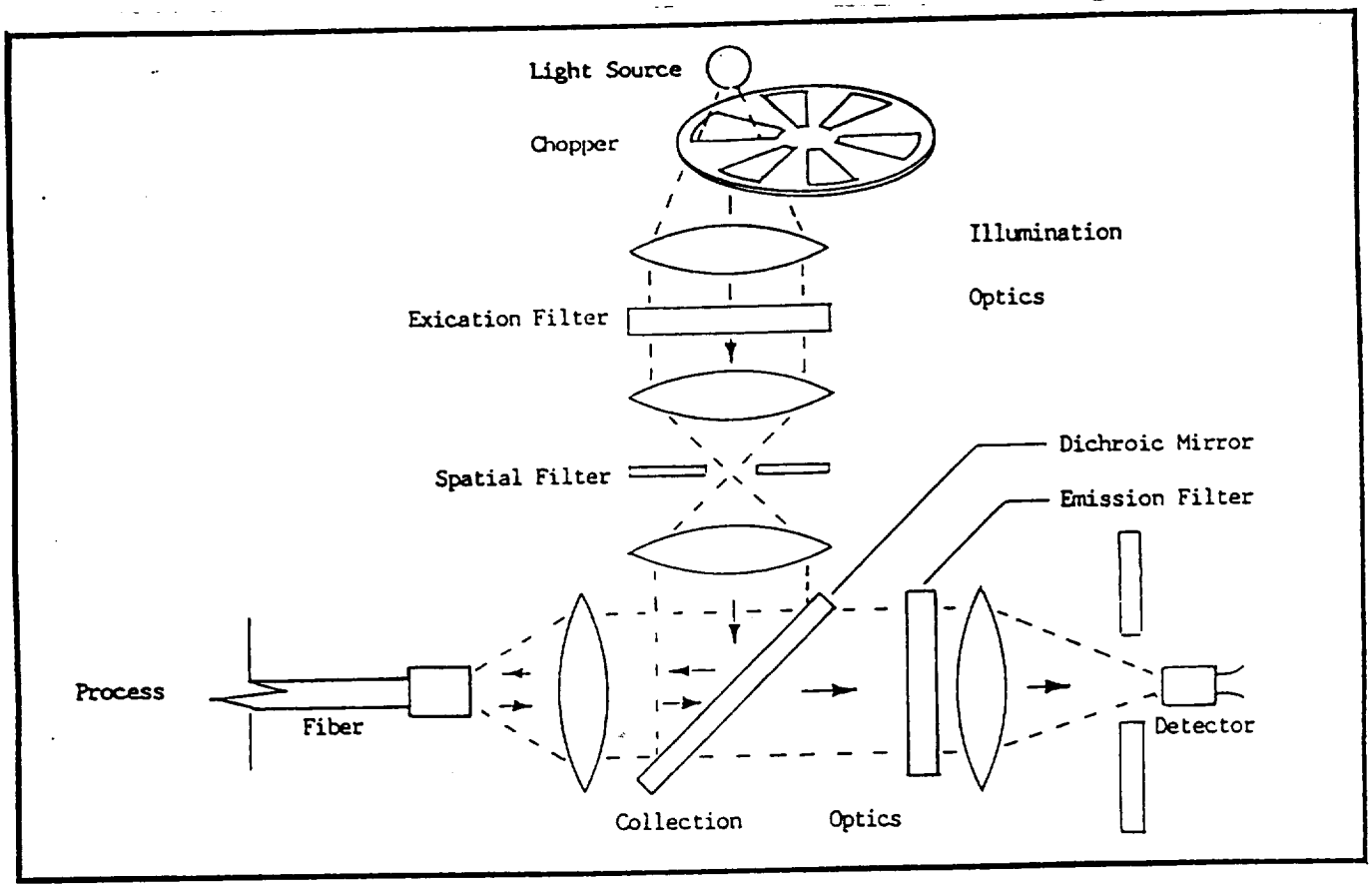
9. Nitrogen laser based time-resolved fluorometers and associated pattern recognition techniques are well defined

Bacteria detection using time-resolved fluorometry can be based on the time-integration of a number of laser pulses so as to achieve extremely high levels of sensitivity. A boxcar average or gated integrator will be used in the proposed instrumentation.

Bacterial identification could be based on the measurement of a series of fluorescence lifetimes over a range of emission wavelengths. In this senerio each type of bacteria could be characterized by a feature vector of decay times stored in memory of the fluorometric instrument. A measurement vector would then be compared with the feature vector library using traditional pattern recognition techniques (e.g. minimum distance classifier, nearest neighbor) to identify the type of bacteria. Using this procedure, total volume analysis is feasible. Typical instrumentation is illustrated in Figure 5.2.

Table 5.2
Excitation/Emission Maxima for Various Biomolecules

Biomolecule	Excitation Peak (nm)	Emission Peak (nm)
Tyrosine	275	303
Tryptophan	287	348
Serotonin	295	330
Phenylalanine	260	282
NADH-NADPH	340	460
ATP, ADP	272	380



Basic Fiber Fluorometer

Figure 5.2

5.3 Secondary (aided) Fluorescence

This proposed methodology would utilize a fluorometer similar to the instrument previously described (Section 5.2). A 100 mL sample would be collected and added to a reaction mixture containing an appropriate non-fluorescing substrate, such as B-Naphthyl-B-D Glucopyranoside or other fluorescently tagged substrate. If viable bacteria are present which can utilize the substrate, the B 1-4 linkage is cleaved, releasing the highly fluorogenic B Naphthyl compound into the medium. The density of bacteria can then be calculated based on the increasing concentration of fluorophore detected over time. By limiting the nitrogen sources available for growth, uncontrolled bacterial growth can be avoided. Concentrating samples by filtration prior to their addition to the reaction mixture will greatly increase sensitivity and allow for liquid, solid and gaseous samples to be processed.

A second concept would simply involve the addition of a 100 mL sample to a reaction mixture (cocktail) containing a fluorophore, i.e. pyrene buteric acid (PBA). This solution is then counted for fluorescence particles of a certain size range. Use of a fluorophore like PBA, in which the bound and unbound molecules have significantly different decay times, eliminates the background interference of the dye. This method could be easily modified to mimic the direct epifluorescence filter technique (DEFT), the direct viable count (DVC) or the microcolony epifluorescence microscopic (MEM) methodology to determine total bacterial numbers or viable microorganisms.

Because of the required addition of a fluorophore, this methodology is best accomplished by processing a collected sample rather, than a fluorogenic molecule being added directly to the storage tanks. Using this procedure, total volume analysis is not possible. Sample size and number are important concerns. The flow cytometric techniques described above are directly applicable to water sources. Air and surfaces samples may be analyzed by transfer to an aqueous medium.

5.4 Volatile Product Detection/Membrane Filtration

The proposed methodology will utilize a modified membrane filtration procedure and will couple microbiological monitoring with specific chemical analysis. By thoughtful consideration of the requirements, a method was devised that will conserve water needed for sampling, meet the microbial requirements and also monitor specific volatile chemical parameters.

The proposed technology for organic chemical detection and analysis is based on a hyphenated mass spectroscopy technique (MS/MS). In particular, a newly developed Teledyne CME Chemical-Biological Mass Spectrometer (CBMS) is proposed as one choice for the detector. A prototype CBMS is being developed by Teledyne CME (Santa Clara, CA) and Bruker-Franzen Analytik (West Germany) for the U.S. Army Chemical Research, Development and Engineering Center (CRDEC). This mass spectrometer will operate automatically to detect, identify and quantitate chemical and biological threat agents at sub-toxic concentration in ambient air. The CBMS Demonstration Model claims to be the most sensitive mass spectrometer in existence today. It is capable of observing material with femtogram/second sensitivity. The CBMS will weigh less than 40 pounds and is designed to be used by ordinary soldiers under battlefield conditions. It uses Quadrapole Ion Storage device (QUISTOR) to perform tandem in-time MS/MS. In contrast to existing "Ion Trap" Technology, the CBMS does not use any extraneous carrier gas, and gives mass peaks with substances present in the air at ppb concentration levels. A demonstration Model was delivered to the CRDEC in 1988.

The Demonstration Model is mounted in an eight cubic foot frame (two feet per side). External to this frame is a 24 V power supply, and a PC/AT computer terminal and screen. In the follow-on work for the CRDEC, the size of the entire unit will shrink to 4 cubic feet with a mass of 40 pounds or less. This Concept Model will utilize an object-oriented, knowledge-based dual computer system, so that the human attendant (possibly wearing bulky gloves and gas mask) will only have four to six large buttons to push. The CBMS will communicate with the outside world using a small screen, a horn, and a serial port for optional telemetry. A Concept Model is scheduled for delivery in November, 1990. It should be noted that similar detectors are currently being developed by Finnigan, The Center for Microanalysis and Reaction Chemistry at the University of Utah and the Oak Ridge National Laboratory (ORNL) for detection of chemical hazards and explosive material.

The proposed procedure would include a 100mL sample being collected in a specialized monitoring device with the sample first pulled through a 0.45 micron filter. The filter is housed within a self contained unit which consists of the filter, nutrient pad, and a sample reservoir. Once the sample is collected, the filter housing is separated from the reservoir chamber and sealed. The sample reservoir is now available for chemical analysis. The filter housing is incubated and periodic head space analysis is conducted using the mass selective detection method. A change in the concentration of the gasses present in the head space indicate microbial activity. The time required to detect changes and the subsequent rates in the monitored atmosphere will indicate the

concentration of the microbial contaminants. The composition of the gasses evolved will also give a preliminary indication of the microbial species present.

The filter housing assembly can be incubated and monitored as necessary. This feature will provide "after the fact" verification by standard methods and will also provide a mechanism for recovery of viable cells in concentrations sufficient for precise identification or antibiotic sensitivity testing. The filter housing will be designed such that it will allow access for manipulation of isolates on orbit, or it can provide short term storage of isolates for their return to Earth.

The choice of media (nutrients and selective agents), which are impregnated onto the pad will determine the specificity of the microbial counts. This system could be modified to provide total counts, gram negative organisms only, gram positive organisms, fecal coliforms, anaerobes, or other specific physiological groups.

Surface samples will be processed and analyzed in much the same manner as described for aqueous samples. A swab of the area in question is made with a specialized pad. The pad is placed in an analysis container, saturated with an appropriate growth media and head space analysis conducted over time. The time required for a change in the head space gasses coupled with their increasing concentration over time will relate to the contamination level. Again this technique may be modified by media and incubation conditions to include heterotrophic counts, coliform counts, Gram-negative bacteria, Gram-positive bacteria

and anaerobic bacteria. Air samples may be analyzed directly by the CBMS detector. For particulate air samples, micro-cascade impaction technology is used to concentrate the particulate contamination of a particular size range. This device, at present, has a quoted sensitivity of 1-10 bacteria per liter of air and **60 virus particles per liter of air.** The capability of this methodology to detect virus particles offers a potential for near real time viral monitoring.

5.5 Surface Acoustic Wave (SAW) Detector

It has been demonstrated that piezoelectric quartz crystals, coated with various polymeric substrates, can be used as highly sensitive detectors for molecules both in the gas phase and in solution phase. In a surface acoustic wave (SAW) device, radio frequency vibrations are induced in a coated crystal and an uncoated reference crystal. When the crystals are coupled, an audio (or acoustic) frequency is obtained from the frequency difference between the two crystals. Absorption of a contaminant by the polymer film of the coated crystal causes a change in the vibrational frequency of the coated crystal and thus produces an easily detectable change in the observed audio frequency. Although a particular coating is generally not perfectly selective (i.e., it will absorb, to some extent, many contaminants), the coating can have a characteristic pattern of absorbances for a series of contaminants. Thus if several different coated crystals (each with its own pattern of absorbances) are used, individual contaminants can be identified by computerized pattern recognition.

Advantages of the SAW approach are simplicity, lack of moving parts, sensitivity (picograms of absorbant can be detected), small size, low cost, and ruggedness. Although it is doubtful that SAW devices will ever achieve the power and versatility of GC/MS, it is entirely reasonable to expect that a number of small, inexpensive SAW devices could be distributed throughout an area to serve as warning monitors for various classes of compounds or specific microbial contaminants.

We have recently shown that gas-liquid chromatography (GLC) can be used to evaluate the selectivity of polymer absorbants to be used in SAW detectors (both GLC retention time and SAW response are dependent on partitioning of the contaminant between gas and polymer phases). Similarly we have developed a method to evaluate polymer selectivities which depends on measurement of the UV-visible absorption spectra of a series of selected dyes in polymer films. Also, we have been involved in synthesis of new polymers which are designed to show selectivity for absorption of various contaminants, and we have been active in surface linkage of polymers to a variety of surfaces.

Initial studies have demonstrated the potential that the SAW technique may be applicable to microbial detection. This technique was first envisioned as a potential biofilm monitor for clean water systems, i.e. ground water, laboratory water. etc. With proper modification, however the usefulness of SAW technology may be applied to microbiological monitoring in Space environments. Two approaches appear plausible. In the first approach we would use our experience in binding proteins to surfaces to couple specific antibodies to the piezoelectric crystals. Binding of a microbe then would produce a readily detectible signal. Keys to success of this technique are availability of monoclonal antibodies for surface antigens and a low degree of nonspecific adsorption onto the immobilized antibody.

In the second approach, specific polymers are coupled to the surface of the piezoelectric crystal. As the polymers are degraded by microorganisms the change in mass is readily

detected. This would eliminate problems associated with nonspecific adsorption. The polymers would be expected to be stable to chemical degradation in a nearly neutral aqueous medium, thus avoiding this possible artifact. As a first step we will couple a variety of polysaccharides to the surface. These materials are readily consumed by a variety of microbes. It would also be of interest to couple proteins to the crystals. In this approach it would be important to characterize the mass-loss patttern for specific microorganisms. Polymers and coatings which demonstrate a high degree of selectivity for attachment and/or consumption by specific organisms or physiological groups will be screened.

6.0 EXPERIMENT DESCRIPTION

6.1 Background

One of the major factors that induced our interest in the development of this project was the need for a sensitive, rapid, reliable microbiological monitor in order to meet the requirements for microbiological specifications aboard Freedom and other long duration missions. We felt that there was a need to investigate the feasibility of employing relatively non-conventional methodologies for microbiological monitoring in Space as well as to study the possibility of modifying existing conventional methods for this purpose. To this end, we have examined approximately thirty different methodologies and evaluated them for application as a microbiological monitor for Space vehicles. In addition, we have investigated the modification of conventional microbiological monitoring methods for their application in Space. These efforts have resulted in the initial definition of six potential candidate methodologies which were further screened for engineering and feasibility criteria. The resultant five candidate monitors are recommeded for further comparative testing in Phase II bench studies which will evaluate the sensitivity, validity, accuracy and precision in the laboratory. The single "best" monitor will then be integrated in the flight experiment hardware for verification of the method in experiments performed on the mid-deck of the Space Transportation System during flight. We envision two or three flight experiments during which the necessary data will be obtained.

The flight experiments are designed to compare data obtained with the NRTMM with more conventional methods with respect to microbial detection and enumeration in Space. Also, since there have been reports of alterations in bacterial physiology and ultrastructure during growth under microgravity conditions, we felt that it was important to obtain data that will enhance our understanding of these phenomena during the flight evaluation of the NRTMM. A significant change in physiology and ultrastructure of microorganisms due to reduced gravity could seriously alter the capability of detection and control of these organisms. Thus, we have incorporated means to evaluate the responses to antibiotics and the ultrastructure of bacteria obtained during various phases of the growth curve and after antibiotic addition. The results of these experiments will serve to validate the NRTMM as well as expand on previous observations thus increasing our understanding of the effects of a microgravity environment on bacteria.

6.2 Experimental Objectives

The overall objective of the experiments performed on orbit will be to evaluate the performance of the near real-time microbiological monitor in Space and to compare the data obtained with those obtained using more conventional methods. The experiments will include the capability to analyze cultures or water samples collected on orbit by the direct epifluorescence filter technique (DEFT), a direct viable counting (DVC) procedure and/or cultural methods. In addition, the experiment will be designed to verify previously reported changes in antibiotic

susceptibility of Escherichia coli induced by growth in a microgravity environment. Samples collected of cultures monitored in Space will also be fixed and returned to Earth for examination by electron microscopy. The data obtained will allow for the verification for the employment of the NRTMM in Space vehicles as well as expand the knowledge base regarding physiological consequences of growth of bacteria in a microgravity environment and the subsequent control of these organisms. The specific objectives will be to:

1. Evaluate the growth of a culture of E. coli or other bacteria of interest in a closed system with the defined near real-time microbiological monitor and compare the data with that obtained by the more conventional procedures.

2. Evaluate the effect of antibiotic addition on the growth of the culture as determined by the NRTMM, DEFT, DVC and/or cultural methods.

3. Evaluate the effect of growth in the Space environment on the ultrastructure of E. coli before and after the addition of antibiotic by means of transmission electron microscopy.

7.0 MICROBIOLOGICAL TEST BED

7.1 Requirement

Soon after completion of the technical evaluations it became apparant that a microbiological test bed (MTB) would be needed for subsequent bench evaluations as well as in-flight evaluation of the candidate NRTMM. A prototype MTB was designed and constructed during this project. The prototype unit will be used to identify critical parameters in the design of the flight unit as well as to identify operational parameters and procedures necessary to evaluate and compare candidate monitors during Phase II bench testing and in-flight validation experiments.

7.2 Description

The internal fluid handling components of the prototype MTB were constructed from Teflon. This allowed for easy cutting and fitting of plumbing sections during the construction and initial modification. The external structural shell is constructed of aluminum. The unit is equipped with large plexiglas windows for viewing during operation. In addition, the prototype unit is capable of steam sterilization between uses or experiments. A Layout Diagram of MTB components is included as Figure 7.1. Also, a schematic of the MTB is presented in Figure 7.2 The MTB was designed with considerations that it should be versatile, relative modular with respect to the internal components, and it should mimic similar operation to that envisioned for closed loop testing in Space.

Figure 7.1 MTB Layout Drawing

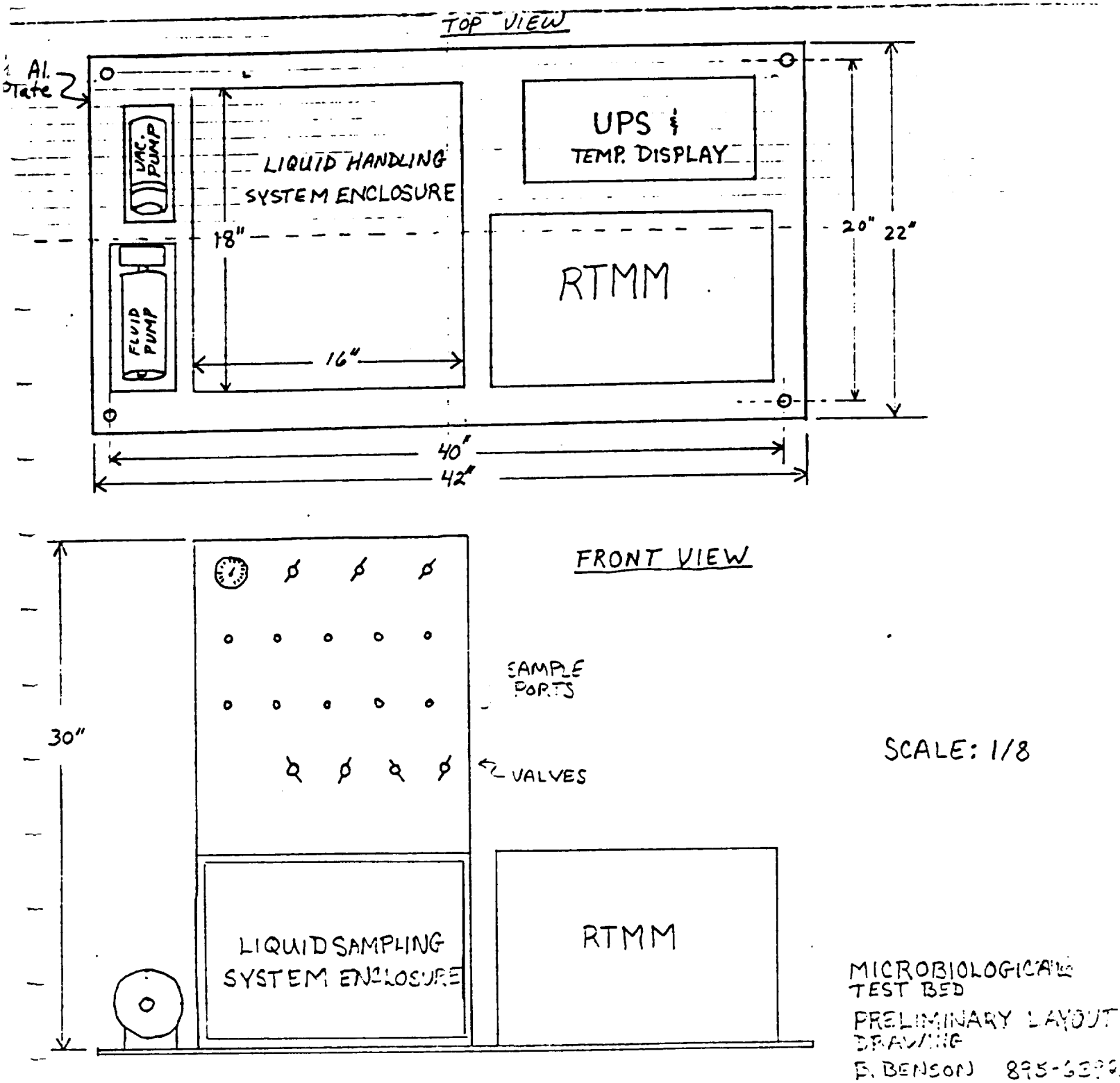
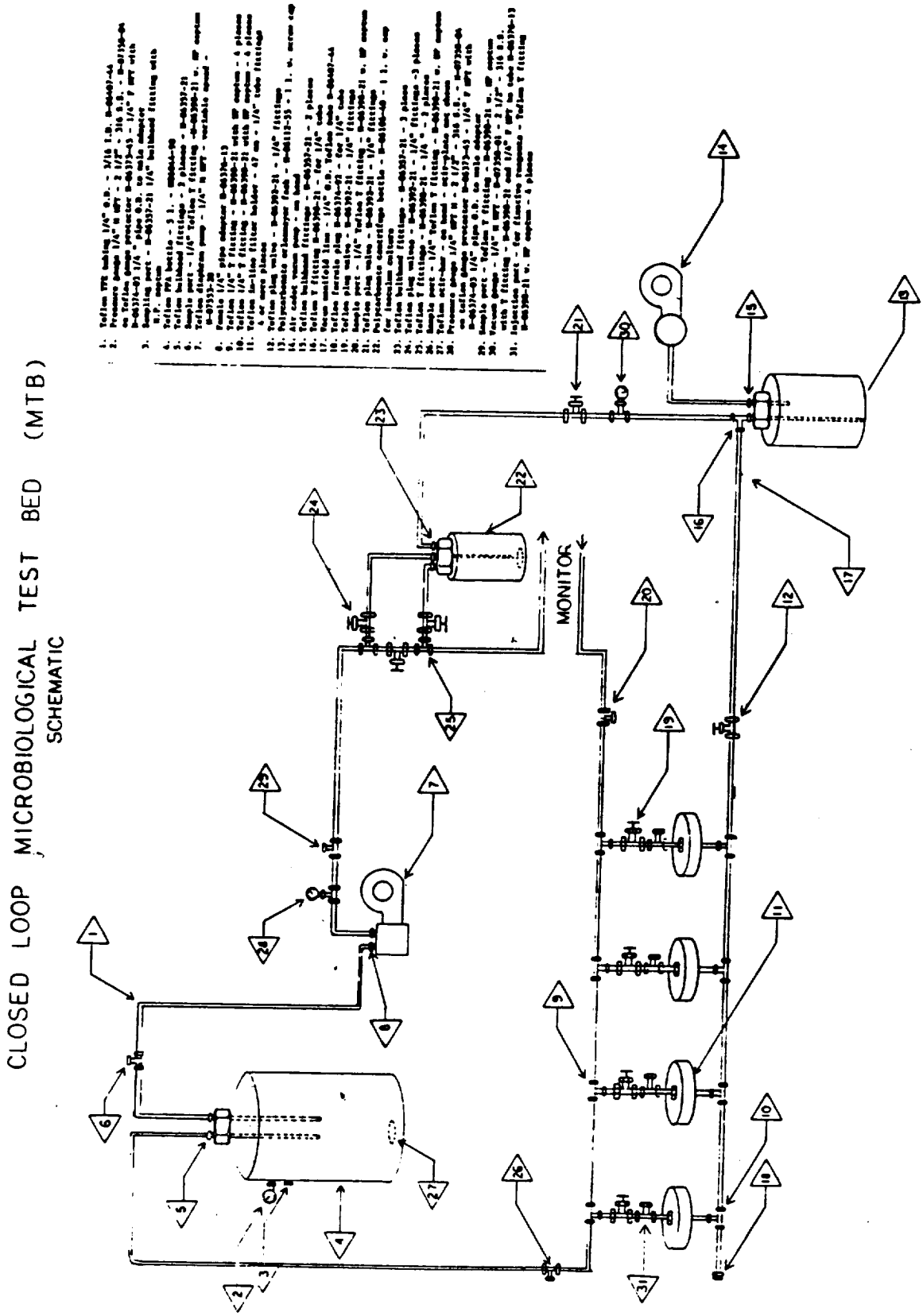


Figure 7.2 MTB Schematic



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The MTB incorporates several features that contribute to its usefulness in evaluating Phase II feasibility of the candidate monitors as well as the types of flight experiments envisioned. Some of the important features incorporated into the MTB design are summarized below:

1. The test bed is a closed loop system that can be interfaced with any candidate NRTMM. Thus, the test bed can be used to evaluate and compare the actual performance of various methods during ground testing and with appropriate modifications can be used to validate the optimal method in Space.

2. The test bed contains a number of semi-automatic filtration sampling ports (11) that can be used to obtain samples either sequentially to get time course types of information or simultaneously to allow for the assessment of different processing procedures or to allow for more accurate statistical treatment of the data. These sampling ports will allow for filtration of samples and any desirable subsequent post-filtration processing of the sample, such as gluteraldehyde fixation, acridine orange staining, etc. It is envisioned that the final flight test bed will contain a set number of automatic filtration sampling ports and a manual sampling port.

3. The test bed provides a preliminary growth chamber (22) and potential dilution chambers (not shown). Ultimately, this will allow for the inoculation of the test bed with a known concentration of bacteria in a defined growth phase.

4. The test bed provides additional (non-filtering) sample ports (3, 6, 20, 26 and 29) that will allow for the acquisition of aseptically collected samples at various locations during the course of ground based testing. These probably will not be incorporated into the final flight apparatus.

5. The test bed is designed such that the entire unit can be placed into an autoclave. This is important to initiate experiments with a sterile unit or if decontamination of the unit is necessary.

Photographs showing the front, side and rear of the MTB are presented in Figures 7.3, 7.4 and 7.5, respectively.

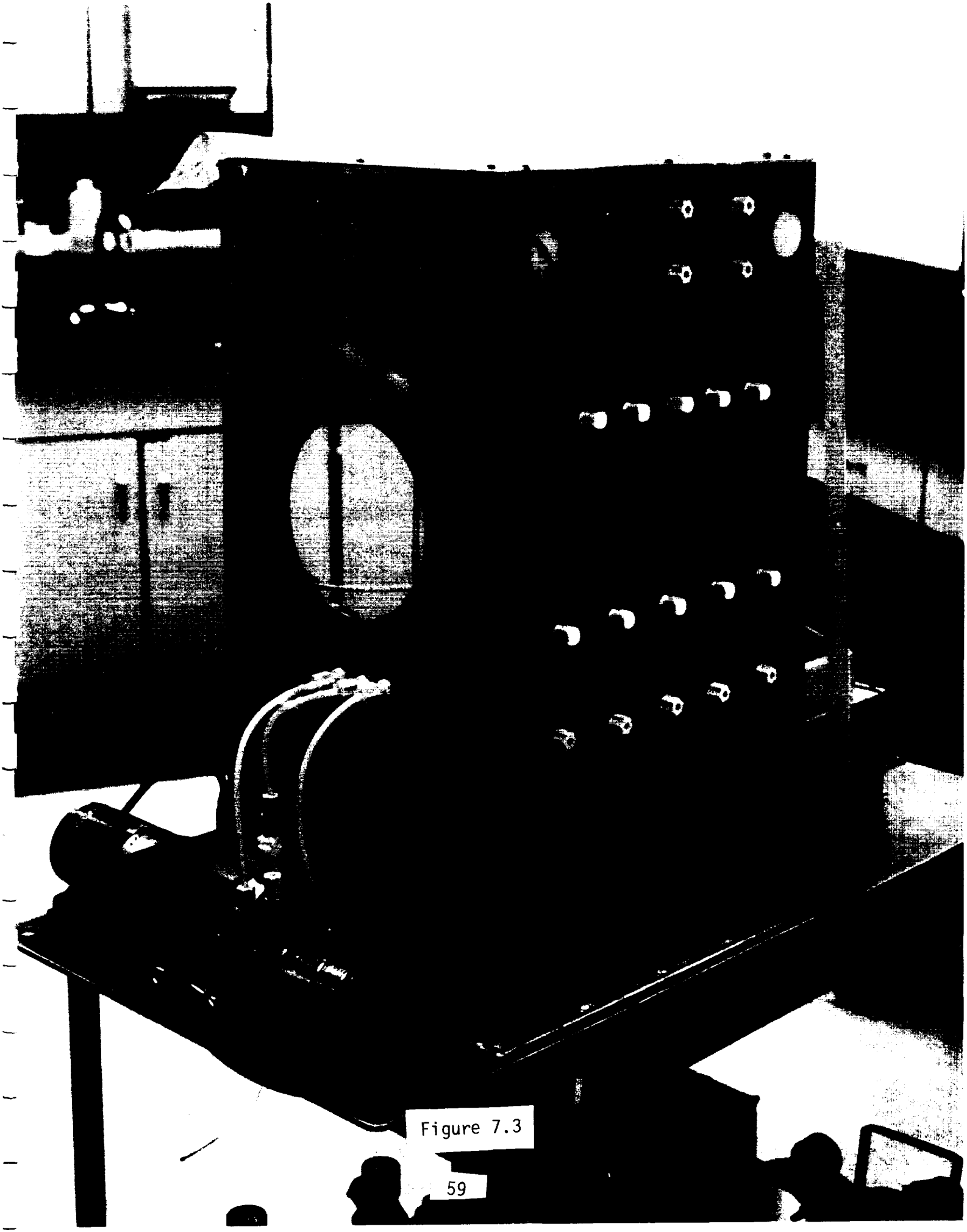

Figure 7.3

Figure 7.4

Figure 7.5

7.3 Ground Based Testing

To date, shake down experiments, defining operational parameters and hardware sterilization have been sucessfully performed. In addition, the MTB was operated for a continuous period of 7 days without detectable contamination as determined by heterotrophic plate counts. In the near future additional testing using the MTB will be initiated to further characterize its operation and performance. A number of potential problems have been identified during the conception and design of the MTB. Some of these experiments are outlined below:

7.3.1 Sterilization and containment of the MTB.

Objective: To define a reliable method to sterilize the MTB.

Experiment 1:

1. Fill the MTB with media.
2. Circulate fluid and manipulate to remove all bubbles.
3. Connect high point of MTB to an open reservoir of media via fluid filled tubing.
4. Sterilize by autoclaving at 121°C for 40 min. (TBD).
5. Allow to cool to ambient temperature very slowly.
6. Open autoclave and immediately close valve between MTB and open reservoir.
7. Assemble pump and attach pressure gauges.
8. Activate pump.
9. Circulate media for 72 hr (TBD), ambient temperature.
10. pull 100 ml of media through each filter sample port and assess each for the presence of heterotrophic bacteria by Standard Methods.

<< OR IF POSSIBLE >>

10. Pull total volume through the filter sample ports (equal volume through each port) and assess these filters for heterotrophic bacteria by Standard Methods.

The absence of colonies on the filters will be interpreted that the sterilizing conditions were achieved and that the MTB is contained.

NOTE: Any of the non-invasive candidate methodologies can be interfaced with the MTB during this test. This is desirable in that it will increase the amount of information obtained.

7.3.2 Bubble formation during operation of the MTB.

Objective: To assess the appearance of bubbles in the lines and compartments of the MTB after sterilization and during operation.

Experiment 2:

1. During the operation of the unit for Experiment 1 the unit will be periodically examined visually for the appearance of bubbles.

If bubbles appear, then the source of the gas for bubble formation will be determined and method(s) to alleviate or eliminate this problem will be defined.

7.2.3 Gas formation during growth of Escherichia coli in the MTB. This will not be a problem in experiments designed to test the performance of the monitor in detecting bacteria in "non-nutritive" solutions.

Objective: To determine whether bubble formation occurs during growth of E. coli in the MTB.

Experiment 3: To be performed in a 37°C walk-in warm room.

1. Sterilize the test bed as per determined protocol. (See experiment 1).
2. Inoculate MTB with E. coli through port (TBD). [Dose TBD]
3. Turn on pump and run experiment for [time TBD] hours.
4. Obtain intermittent samples through both the filtering sample ports and the non-filtering sample ports to evaluate growth during the experiment and to compare the values obtained by each sampling method.
5. Observe the MTB periodically to determine whether bubble formation occurs.

If bubble formation is observed then use alternative bacterial species or defined medium with a substrate that does not result in significant gas production.

NOTE: This experiment also allows for the testing of the filtering sampling ports and comparison of the values obtained with those obtained by an independent method.

NOTE: This experiment can be performed with a candidate NRTMM interfaced to the MTB. This would allow for the comparison of values obtained by the filtering port samples, non-filtering port samples, and the NRTMM.

7.4 KC-135 Flight Evaluation

In addition to the ground based testing using the MTB, microgravity experiments have been planned for the KC-135. The MTB prototype unit was initially manifested to fly during the first week of August, 1989. However, due to a unforeseen structual problem the flights have been postponed until further notice.

Three flights are currently planned during FY-90. The first flight of the prototype hardware will be a microgravity shakedown experiment. During these flights operational parameters will be reviewed and assessed under microgravity conditions. In addition, both conventional and non conventional analysis procedures will be conducted. Candidate NRTMMs will be incorporated and evaluated for performance as well.

Appendix C contains the supporting documents which are applicable to the planned KC-135 flight experiments. This documentation includes the request for microgravity test support, test equipment data package and associated hazard reports.

At the present time, four experimenters in our laboratory are certified to fly on the KC-135.

7.5 STS Flight Evaluation

It is envisioned that a similar version of the prototype MTB will be a necessary part of the flight experiment hardware planned for Phase II in-flight evaluation. Design modifications identified during the bench evaluations and the KC-135 flights will be incorportated into the flight unit.

8.0 MODIFICATION OF STANDARD ENUMERATION TECHNIQUES

At the present time, only cultural methods are available which meet the current specifications set for Space Station Freedom. First, as defined, the specifications indicate requirements based colony forming units which dictate the use of cultural methods. Cultural methods do represent a mechanism for the direct determination of viable and cultural microorganisms. The sensitivity of the method employed is based on the volume of sample to be analyzed. The drawbacks to culturable methodologies are primarily they are both labor and time intensive. In addition, they require the growth of microorganisms within the Space Station environment. In support of these methods they 1) have a proven track record on Earth, 2) should be readily adaptable to the Space Station environment, 3) are readily adaptable to various physiological groups, and 4) meet the current water quality specifications. It is possible that the time required for conventional analysis of these parameters coupled with the longer duration missions and the production of potable and hygiene water in-flight might prove to be unacceptable in assuring the health maintenance of the crew and the reliability and proper operation of the various systems and subsystems associated with environmental control and life support.

Recent advances in the assessment of microbiological quality on Earth may have application to the Space Station environment. The development of a direct epifluorescence filter technique (DEFT) for determination of total bacterial numbers can provide rapid information regarding microbiological quality and has been

identified as a likely candidate for Space application. The method requires only two-three hours to complete but requires moderate crew involvement and interpretation and does not differentiate between viable and non-viable cells.

The second method is a direct viable count (DVC) procedure. In this procedure, bacterial cells are incubated in the presence of naladixic acid to prevent cell division. Following incubation, acridine orange is used to stain the cells and only the enlarged cells are counted as substrate responsive or "viable". Non responsive or "non-viable" cells remain normal sized. The third method includes modified membrane filtration procedures which incorporate a fluorogenic substrate in the medium. With bacterial growth and assimulation of the substrate a fluorescence micro-colony is produced that is rapidly detected. This procedure requires approximately 7-8 hours and at the present time is only applicable to coliform organisms. Laboratory studies were initiated to evaluate the potential for miniaturization and modification of standard enumeration techniques for space applications. These included 1) a laboratory study evaluating the direct miniaturization of the heterotrophic plate count (HPC) procedure, 2) a second study initiated to compare the miniaturized HPC procedure to an epifluorescence technique and 3) a study to modify the direct viable counting procedure. Each of these studies, as well as other potentially suitable methods are discussed below.

8.1 Miniaturization of Membrane Filtration (MF) Technique

In order to use membrane filtration techniques for Space applications the procedure and associated hardware and expendibles must be miniaturized. To assess the feasibility of miniaturizing the procedure two basic studies were initiated. First, flow studies were conducted in order to assess the volume limitations which result from the reduction in filter surface area. Results indicated that volumes and flow rates using 13 mm filters were unsuitable to obtain the required sensitivity. In addition, 25 mm (0.22 u) filters were demonstrated to handle an optimum volume of 60 mL (99% CL) and maximum volumes not exceeding 200 mL (Figure 8.1). In comparison, 47 mm (0.22 u) filters were demonstrated to handle an optimum volume up to 400 mL (99%CL) and maximum volumes approaching 1 L (Figure 8.2).

A second study was initiated to demonstrate the comparability of 25 and 47 mm filters for standard membrane filtration techniques. In this study, good correlation was obtained between filter sizes. In addition, it was determined that countable ranges were directly related to filter area, as suspected, and that the range of 20 to 50 CFUs could be easily counted on 25 mm filters. Furthermore, by incorporation of special filters with hydrophobic grids, the counting range obtainable using the smaller diameter filter membranes can be increased further.

Flow Rates
25 mm Filters

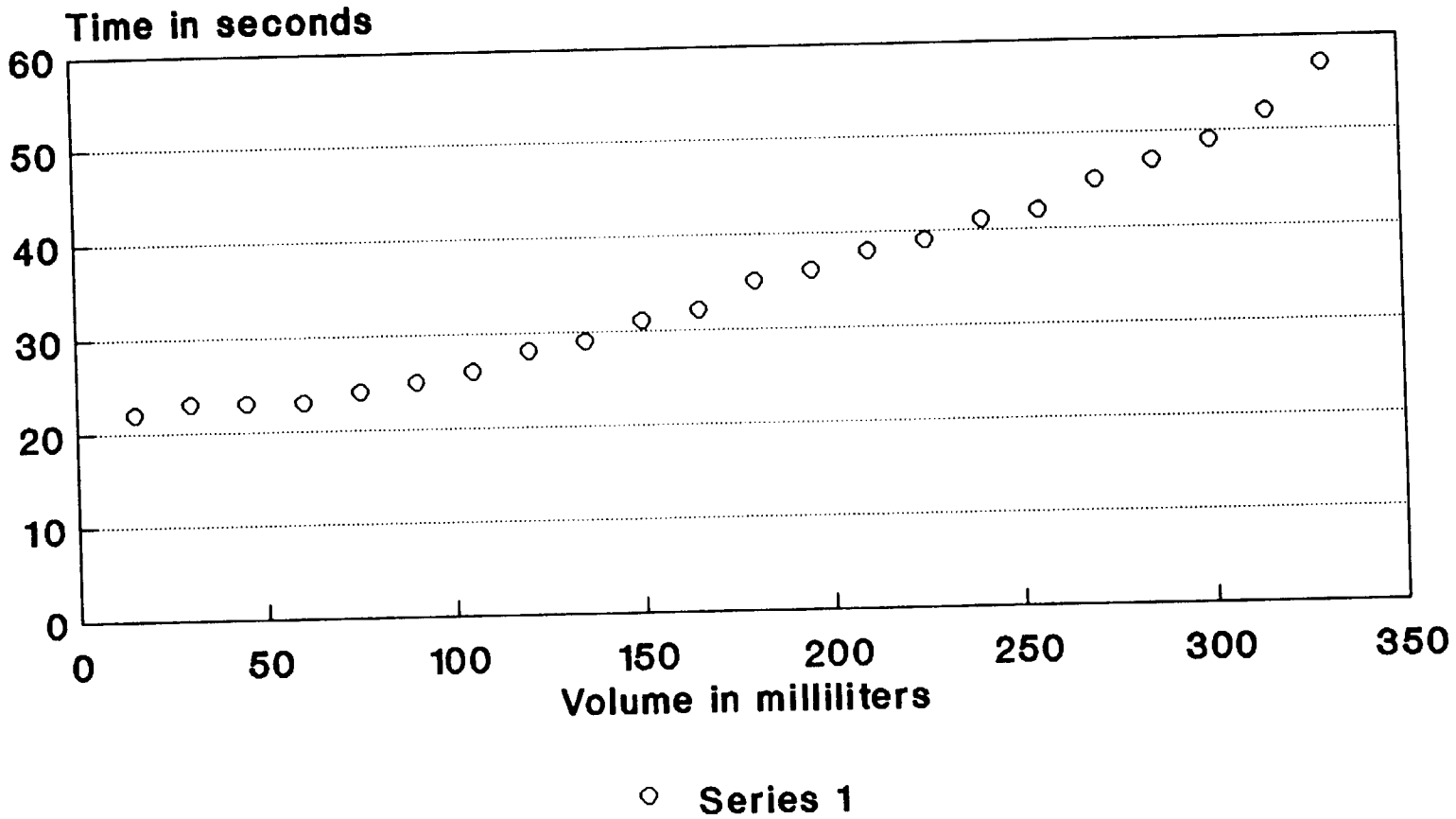

Figure 8.1

Flow Rates
47 mm Filters

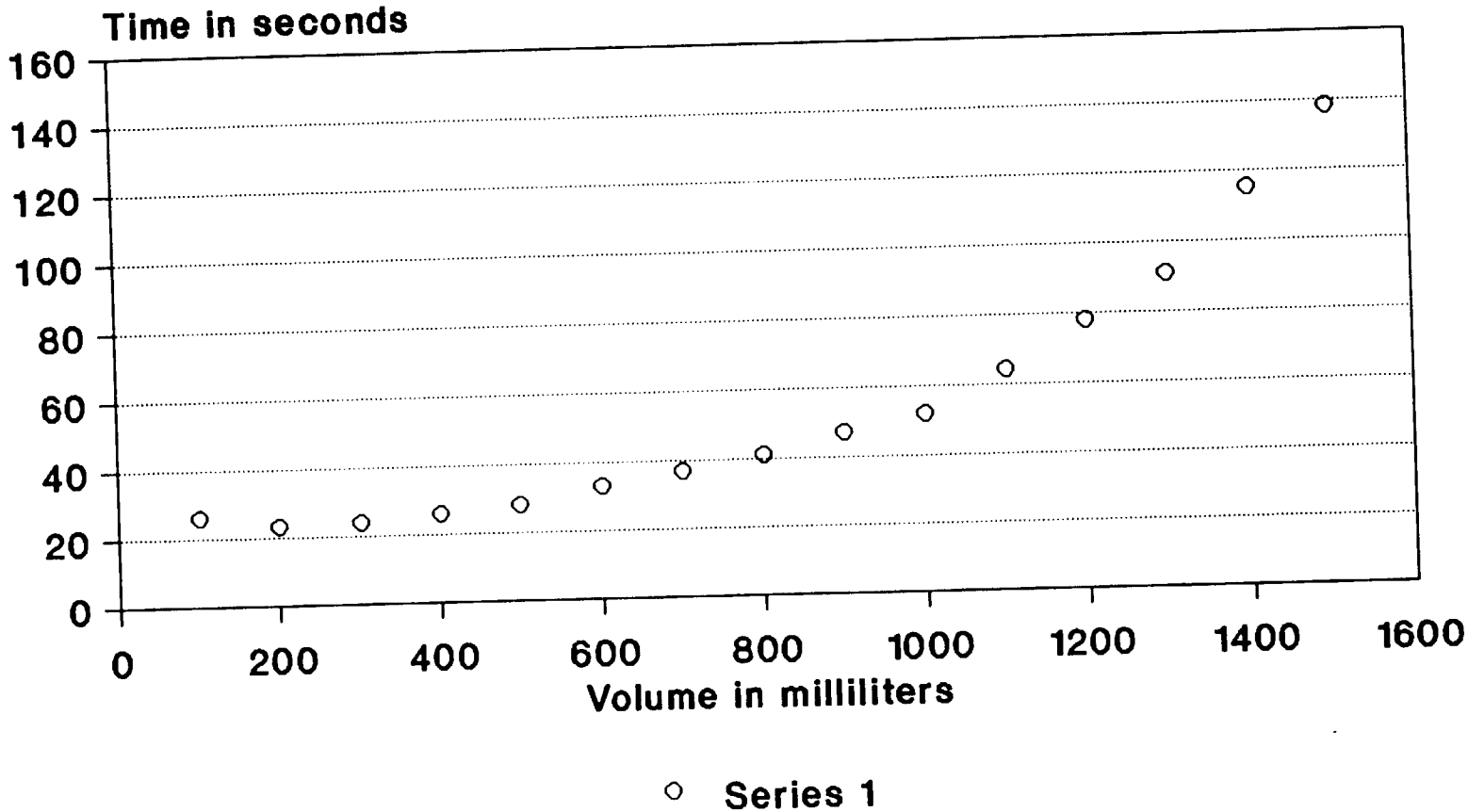

Figure 8.2

8.2 Comparison of Epifluorescence and MF Techniques

Much debate currently exists between the use of direct counting procedures and viable plate counts. The realization that viable but non-culturable microorganisms are frequently encountered both from low nutrient (starved) and other high stress conditions, as well as the limited growth potential which many organisms possess, further illustrates this as a serious problem. Other studies have shown that electron microscopy counts are higher than the direct epifluorescent filter technique (DEFT) which are higher than direct viable counts (DVC) which are higher than viable (heterotrophic) plate counts (HPC). Initial studies conducted in our laboratory using log phase cultures did not show this trend (Table 8.1). Although these results were somewhat unexpected, it is reasonable that log phase cultures grown on a non-selective media would not markedly demonstrate this phenomenon. Additional studies have demonstrated that marked differences do exist in stationary phase cultures and to a greater extent from environmentally derived samples. In fact, the differences in comparability are source related, i.e. the greater the environmental stress the less comparability exists between the aforementioned techniques. One final note is the observation that considerable differences exist in the counting of cells using the direct epifluorescence filter technique (DEFT) between analysts and it was even difficult for untrained analysts to differentiate between cells and inanimate debris. This variable alone would probably account for an excessive number false positive responses from different and untrained crew personnel.

TABLE 8.1
Comparison of Viable Plate Counts, AODC and DVC Methods
Using log phase cultures

Culture	MF Viable Plate Count (CFU/mL)	AODC (cells/mL)	DVC (enlarged cells/mL)
K. pueumoniae	4×10^8	4.6×10^8	4.2×10^8
E. Coli	1.3×10^8	1.8×10^8	1.2×10^8
B. alvei	7.5×10^5	1.0×10^6	5.9×10^6

8.3 Direct Viable Counting Procedure

Due to the growing concern of viable but non-culturable bacteria, the direct viable count (DVC) is gaining widespread popularity. This procedure was not one routinely conducted in our laboratory and it was felt that it was important to evaluate. The DVC method was set up, optimized and compared to both the DEFT and MF procedures. Using this method estimations of viable cells can be made independent of identifying all culture and nutrient requirements of the population. Thus, a better estimation of substrate responsive (viable) bacterial densities can be obtained. In these studies it was demonstrated that samples could be rapidly processed using this method. However, the procedure requires approximately 12 hours incubation for optimum results. Many of the cell/debris problems noted previously were eliminated since only enlarged cells are counted. In addition, the results compared favorably to DEFT and MF (HPC) values using log phase cultures (Table 8.1). One major disadvantage is the limitation of sample size which can be used

in this procedure. Additional studies were conducted to modify the DVC methodology to a membrane filtration (MF) technique in order to increase the sensitivity to that obtainable with the standard MF viable plate counts with promising results. Modifications of this procedure can be incorporated into a secondary fluorescence, electrical impedence or laser light scattering method which will potentially eliminate the need for microscopic observations.

8.4 Microcolony Epifluorescence Microscopy

The microcolony epifluorescence microscopy (MEM) method involves the concentration of bacteria and/or fungi through a membrane filter and transferring the filter to a selective medium. The filters are then incubated for 3-6 hours after which the bacteria are fixed with formaldehyde and stained using acridine orange. The microcolonies are then counted using epifluorescence microscopy. A wide variety of media can be used including both selective and non-selective formulations. Multiple steps for the recovery of stressed or injured bacteria may also be incorporated into this procedure.

8.5 Rapid Plate Counts

Several rapid plate count procedures have recently been described. These procedures use a fluorphore indicator incorporated into the medium for the rapid detection of microcolonies. To date, this technique has only been evaluated for select physiological groups but could reasonably be modified for other microorganisms. The advantages of this procedure is that only culturable microorganisms are enumerated and that

viable cells are available for archiving or further evaluation. This procedure requires approximately 7-8 hours for gram negative bacteria. No data was available for gram positive bacteria or fungi.

8.6 Multiple Tube Fermentation

The multiple tube fermentation procedure can be modified to enumerate heterotrophic bacteria. The results of replicate tubes and dilutions are reported in terms of the most probable number (MPN). The the determination of bacterial density in a sample is based on Poisson distribution. Using this procedure, sample size is limited but recovery of injured or stressed organisms is generally higher than comparative plating techniques. Sensitivity of the procedure can be effectively increased by the addition of a fluorogenic compound to the culture medium. Gennerally, multiple tube fermentation procedures can be developed for specific physiological groups of interest.

8.7 Summary

With the development of a rapid monitoring technique for microorganisms, the importance of rapid standard techniques are obvious. They will prove to be valuable in verification of the rapid monitoring technique, for terminal characterization and as a contingency (backup) method for the NRTMM.

At the present time, it is noted that the DEFT does provide valuable information regarding the total bacterial numbers present in a sample. In addition, with the proposed image analysis equipment proposed for Freedom the disadvantages of crew

evaluations in-flight are minimized. However, if a problem is noted using the DEFT little information is gained regarding the physiological groups present or the viability of the cells. In these instances follow-up cultural procedures must be used to obtain this information. Furthermore, the use of remote image analysis processing on longer duration Space flight and planetary bases probably has limited application. For these reasons, it is imperitive that reliable and rapid cultural procedures be developed. Ideally, a rapid culture procedure for the various physiological groups of interest is the method of choice. In this case, further analysis of the contaminant (identification and/or antibiotic suceptability) and archiving can be accomplished. The second method of choice would be the MTF/MPN procedure provided that the required detection limits can be obtained. This method would provide for subsequent follow-up characterizations (identification and/or antibiotic sensitivities) and contaminant archiving but would first require primary isolation as an additional step. Third, would be the MEM procedure. However, only verification of the density and presence of certain physiological groups are possible. Information regarding speciation or antibiotic suceptability cannot be gained from this procedure. Also, archiving the contaminant is not possible. Other methods such as standard plate count and DVC procedures are not recommended for application in Space.

Regardless of the conventional method chosen for verification and validation in Space, concentration of the sample through a membrane is ideal to meet the required specifications. Due to the volume requirements necessary the smallest diameter filter which can be effectively used is approximately 25mm. Optimization of filter types for flow, pore size and recovery will be a focal point in the Phase II efforts.

9.0 OTHER EFFORTS

9.1 Presentations and Publications

During the contract period other related efforts included the preparation of a presentation given at the OAST Workshop held in Atlanta, Ga, December 6-9, 1988 and the preparation of a manuscript and presentation given at the 19th Intersociety Conference on Environmental Systems held in San Diego, Ca, July 24-26, 1989. A copy of each of these are included in Appendix D.

9.2 Subcontracts

A subcontract to Dr. Kenneth Schlager, Biotronics, Inc. (Wauwatosa, Wisconsin) was partially funded from this contract. Dr. Schlager's efforts concentrated on the review of spectroscopic methods which could potentially have application to near real-time microbiological monitoring. Dr. Schlager's efforts and recommendations have formed the basis for the conceptual design slated for the instrumentation required for primary and secondary fluorescent detection. A copy of Dr. Schlager's progress reports are included as Appendix E.

10.0 BUDGET SUMMARY

A budget summary is presented in Table 10.1 illustrating the project expenditures which correspond to these efforts.

TABLE 10.1

FINANCIAL REPORT SUMMARY

CONTRACT: FNAS Microbial Monitor

ACCOUNT NUMBER: 5-31886

REPORT PERIOD: 6/1/88 - 8/31/89

		Budget	Expended	Encumbered	Balance
I.	Salary & Wages	43,996.00	39,550.60	0.00	4,445.60
II.	Fringe	8,635.00	6,794.58	0.00	1,840.42
III.	Operating Expenses	16,525.00	9,194.14	0.00	7,330.86
IV.	Laboratory Supplies	0.00	5,948.77	125.24	<6,074.01>
V.	Travel	0.00	3,837.53	0.00	<3,837.53>
VI.	Facility Useage	14,634.00	14,634.00	0.00	0.00
VII.	Indirect	21,670.00	27,378.91	53.85	<5,762.76>
	TOTAL:	105,460.00	107,338.53	179.09	<2,057.62>

Date prepared: 10/2/89

Prepared by: M.V. Kilgore, Jr.

APPENDIX A

METHODOLOGIES

1.0 Viable Plate Count/Membrane Filter Technique

CONCEPT: Single Cell Detection

METHOD: Viable Plate Count, Membrane Filtration Technique

DESCRIPTION: A sample is obtained and passed through a membrane filter of appropriate pore size. A 0.2 um pore size is generally used in current water quality analysis, however, we recommend employing a 0.1 um pore size filter. The filter is then placed on either an absorbent pad saturated with an appropriate growth medium or on the surface of an appropriate agar medium.

INSTRUMENTATION:

An automated sampler is desirable.

An automatic colony counter is desirable.

CHEMICAL PARAMETERS (ACTIVE SPECIES): No specific chemical parameters. A positive signal is the formation of a colony on the surface of the filter.

SENSITIVITY: This method can detect a single cell capable of forming a colony on the medium (media) employed.

VOLUME: With the microbial specifications for the Space Station set at present at <1 bacterium/protozoan/fungus per 100 ml of potable water, it is recommended that 500 ml be sampled at each sampling time.

SPECIFICITY: By definition this method even when intended to enumerate "total bacteria" or "total fungi" will detect only those organisms capable of forming colonies on the medium and incubation conditions used. Only true colony forming units will be detected. Will not detect viable but non-culturable bacteria.

INVASIVE OR NON-INVASIVE: Invasive sampling methods would have to be employed.

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-destructive for those microorganisms recovered.

ID POTENTIAL: By employing appropriate selective and differential media can obtain some general identification information.

MANIPULATION: This method would require significant manipulation of the sample, filter, and plating medium.

REVIEW OF APPLICATIONS AND VALIDITY: A wide variety of modifications of the membrane filter technique have been used upon numerous occasions for the enumeration of "total bacteria", specific groups of bacteria, and yeasts in clinical, quality control, field and research laboratories.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. Limited to detection of cells capable of forming colonies on the medium and incubation conditions used.

A. Allows for recovery and archiving of those microorganisms detected.

D. Generates biohazardous waste.

D. Will require significant crew time unless complex robotic analysis instrumentation is developed.

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2.0 Viable Plate Count - Spread or Pour Plate

CONCEPT: Single Cell Detection

METHOD: Viable Plate Count, Spread or Pour Plate Technique

DESCRIPTION: A sample is obtained and passed through a membrane filter of appropriate pore size. A 0.2 um pore size is generally used in current water quality analysis, however, we recommend employing a 0.1 um pore size filter. The filter is then placed on either an absorbent pad saturated with an appropriate growth medium or on the surface of an appropriate agar medium.

INSTRUMENTATION:

An automated sampler is desirable.

An automatic colony counter is desirable.

CHEMICAL PARAMETERS (ACTIVE SPECIES): No specific chemical parameters. A positive signal is the formation of a colony on the surface of the filter.

SENSITIVITY: This method can detect a single cell capable of forming a colony on the medium (media) employed.

VOLUME: With the microbial specifications for the Space Station set at present at <1 bacterium/protozoan/fungus per 100 ml of potable water, it is recommended that 500 ml be sampled at each sampling time.

SPECIFICITY: By definition this method even when intended to enumerate "total bacteria" or "total fungi" will detect only those organisms capable of forming colonies on the medium and incubation conditions used. Only true colony forming units will be detected. Will not detect viable but non-culturable bacteria.

INVASIVE OR NON-INVASIVE: Invasive sampling methods would have to be employed.

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-destructive for those microorganisms recovered.

ID POTENTIAL: By employing appropriate selective and differential media can obtain some general identification information.

MANIPULATION: This method would require significant manipulation of the sample, filter, and plating medium.

REVIEW OF APPLICATIONS AND VALIDITY: Various modifications of the membrane filter technique have been used upon numerous occasions for the enumeration of "total bacteria", specific groups of bacteria, and yeasts in clinical, quality control, field and research laboratories.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. Limited to detection of cells capable of forming colonies on the medium and incubation conditions used.

A. Allows for recovery and archiving of those microorganisms detected.

D. Generates biohazardous waste.

D. Will require significant crew time unless complex robotic analysis instrumentation is developed.

REFERENCES:

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3.0 Direct Viable Count

CONCEPT: Single Cell Detection

METHOD: Direct Viable Count

DESCRIPTION: A sample is passed through a filter, allowing for the entrapment of cells on the filter surface. After a subsequent incubation of the filter with yeast extract and naladixic acid, the filter is stained with acridine orange, much as in normal epifluorescence, the filter is examined for fluorescent filaments.

INSTRUMENTATION:

As described for epifluorescence.

In addition, an incubator is required.

CHEMICAL PARAMETERS (ACTIVE SPECIES): No specific parameter. Cell elongation and subsequent staining with acridine orange provides the signal of interest.

SENSITIVITY: A single filament can be detected.

VOLUME: As described for epifluorescence, the specifications currently imposed may require significantly large volumes in order to obtain meaningful results.

SPECIFICITY: Will detect those cells able to increase in cell mass under the conditions imposed.

INVASIVE OR NON-INVASIVE SAMPLING REQUIRED: This method requires invasive sampling.

DESTRUCTIVE OR NON-DESTRUCTIVE: We have not been able to find published applications of this method where the organisms have been recovered for subsequent culture and analysis. It is likely that the naladixic acid treatment and subsequent acridine orange staining is cidal to the cells that are detected.

ID POTENTIAL: This method has little value regarding identification of the detected microbes.

MANIPULATION: As described with epifluorescence with the addition of an incubation period.

REVIEW OF APPLICATIONS AND VALIDITY: This technique has received much interest recently. The advantage of this technique over epifluorescence rests in the ability to detect an increase in the mass of recovered cells on the filter surface. One relatively common, often misleading, representation of the direct viable count is that all viable cells are detected. This method is more sensitive than the Viable Plate Count/Membrane Filter Technique

with respect to the detection of viable cells in that there is no requirement for the formation of colonies or even of cell division. However, as with any method requiring macromolecular synthesis by the cells, only those cells that can metabolize significantly under the conditions imposed will be detected. For example, it is unlikely that a strict anaerobe would be detected as a viable cell if the incubation took place under aerobic conditions.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Has the advantage of detecting as viable those cells capable of increasing in mass under the incubation conditions.

L. Limited to detection of cells capable of increasing in mass under the incubation conditions imposed. That is to say that a cell that does not increase in mass is not necessarily non-viable.

L. No evidence that spores can be detected as viable cells by this method.

L. It is unlikely that detected cells could be recovered for archiving and further analysis.

D. Generates biohazardous waste.

D. Generates chemical waste.

D. Will require significant crew time unless complex robotic analysis instrumentation is developed.

L. Not applicable to viruses.

D. The relatively involved sample manipulation is disadvantageous in that it requires either a significant dedication of crew time or a significant development in robotics. Experts in robotics should be consulted in order to determine feasabilty and cost regarding the employment of this technology.

D. Analysis of the dyed filters requires either the time of trained individuals or the employment of computer image analysis. Experts in image analysis should be consulted in order to determine feasabilty and cost regarding the employment of this technology.

D. Specific toxic hazards for acridine orange are not available. This chemical is designated as harmful if inhaled or swallowed (see accompanying Material Safety Data Sheet).

L. No evidence that the Aphragmabacteria are detected by this method.

L. No evidence that bacterial or fungal spores or protozoan cysts are detected by this method.

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4.0 Laser Light Scattering

CONCEPT: Single Cell Detection

METHOD: Laser Light Scattering

DESCRIPTION: Particles in suspension will scatter incident light when illuminated. In this method, a laser energy source is employed and light scattering is measured. The intensity of the light scattered can be measured at one or more angles.

INSTRUMENTATION: Laser light source with photodiode detectors, optimally with interface to computer for data storage and analysis.

CHEMICAL PARAMETERS (ACTIVE CHEMICAL SPECIES): Any particle with a refractive index significantly different from the suspending medium will scatter light. No specific chemical parameter detected.

SENSITIVITY: Given that a sufficient difference in refractive index between particle and suspending medium is present a single particle can be detected.

VOLUME: This method would not require the actual removal of a sample volume, since the detection system could be configured to monitor in-line.

SPECIFICITY: There is some indication of specificity of this method with respect to differential scattering spectra among various bacteria ( ). It is questionable at this time whether an automated monitor employing this method could distinguish between inanimate particles and microbial cells. The performance of definitive experiments regarding this is recommended.

INVASIVE OF NON-INVASIVE: This method could be configured to be non-invasive.

DESTRUCTIVE OR NON-DESTRUCTIVE: Low power lasers could be employed to enhance the non-destructiveness of the method. The maintenance of viability should be confirmed by appropriate experimentation.

IDENTIFICATION POTENTIAL: Low; there is some evidence in the literature regarding differential scattering spectra among various bacteria ( ). In addition, it appears that cells in different physiological states show different scattering spectra (P. Wyatt, personal communication).

MANIPULATION: No or very little manipulation is required if configured to perform in-line monitoring.

REVIEW OF PUBLICATIONS; APPLICATIONS AND VALIDITY: Light scattering had shown promise as a physical method to study bacteria early in the development of general bacteriology (Koch., 1981). Turbidimetric mesurements have routinely been used by bacteriologist to estimate cell numbers in liquid media. The development of laser light scattering enhanced the possible applications of this technique to the analysis and detection of microscopic particles. Wyatt (see references) has developed an instrument that will measure scattered light simulataneously at a number of angle relative to the incident light. As mentioned earlier light scattering patterns appear to be somewhat indicative of the particle scattering the light. Some differences between bacterial species and between antibiotic treated bacteria were detected. This method appears to be promising with the limitations discussed below.

REQUIREMENTS FOR CALIBRATION AND INFLIGHT QUALITY CONTROL: Alignment of the laser would be critical. This type of monitor could be calibrated with standard particulates in suspension.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Rapid detection of particulates in sample.

A. Can be configured as a non-invasive method.

L. Capability to distinguish animate from inanimate objects limited.

L. Lifetime of lasers is limited and thus a routine changeout schedule will be required. However, some lasers have as long an expected lifetime as 10,000 hrs.

D. Power requirements may be restrictive.

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5.0 Primary Fluorescence

CONCEPT: Single cell detection

METHOD: Primary Fluorescence

DESCRIPTION: Fluorescence of naturally occurring microbial components is determined. Most commonly a suspesion of cells is monitored.

INSTRUMENTATION:

Fluorometer (specs to be deterimined) with interface to computer.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Fluorescent components of proteins, nucleic acids, and pyridine nucleotides as well as some fluorescent pigments.

SENSITIVITY: Lower limit of sensitivity is unknown.

VOLUME: No volume removal required.

SPECIFICITY: With the employment of primary fluorescence, it should be possible to distinguish between animate and inanimate materials.

INVASIVE OR NON-INVASIVE: Unless instruments providing a signal to noise ratio great enough to detect a single cell are identified this method will have little applicability to a real time monitor where continuous monitoring of the flow stream is employed. For application to subsequent supportive identification instrumentration, this method should be given consideration.

DESTRUCTIVE OR NON-DESTRUCTIVE: Depending on the wavelength and power of laser required there is potential for this method to kill microorganisms.

ID POTENTIAL: Has demonstrated limited identification potential to the genus level for the studies published regarding emission or excitation spectra (Dalterio, et. al., 1986, Dalterio et. al., 1987). Decay characteristics of primary fluorescence, however, appears to be more promising regarding identification (Dalterio, et. al., 1986, Dalterio, et. al., 1987).

MANIPULATION: Sample manipulation that would be required if employed in the invasive configuration would be minimal. A concentration step via filtering or centrifugation may be required prior to analysis.

REVIEW OF APPLICATIONS AND VALIDITY: A number of variations of employing UV primary fluorescence for the detection and identification of microorganisms have been described. This method appears to have promising application to the detection of certain bacteria that synthesize fluorescent pigments. For example, certain species in the genus Bacteroides can be differentiated on the pattern of colonial fluorescence (Slots & Reynolds, 1982). One can envision potential application to single cell detection of species of bacteria that contain a significant amount of such fluorescent pigments. More generally, as stated earlier a number of compounds more distributed throughout the biological world as important cellular components, such as ribo- and deoxyribo- nucleotides, flavins, pyridine nucleotides, and aromatic amino acids, display significant fluorescence and may provide a detectable signal if the optical systems can be developed to accomplish this goal. Primary fluorescence has been applied to the study of microorganisms in four major ways:

1. Fixed excitation and emission wavelength fluorescence determination. This includes in some cases the determination and analysis of fluorescence decay curves (Dalterio, et.al. 1986, Dalterio, et.al. 1987). By measuring emission at varying excitation frequencies varying excitation maxima have been reported. For an emission wavelenth of 450 nm, Staphylococcus epidermidis, Enterobacter cloacae, and Pseudomonas fluorescens all exibited excitation maxima at between 380 and 400 nm. For emission wavelength of 340 nm, both S. epidermidis and E. coli exhibited an excitation maximum at 290 nm. Emission maxima were observed at From the data available is felt that optimal excitation and emission wavelengths could be identified in order to use this method to detect microorganisms. The application of fluorescence decay for bacterial identification has been suggested by Nelson and coworkers (Dalterio et. al., 1986, Dalterio et. al., 1987).

2. Determination of emmission spectra at fixed excitation wavelength. These spectra have been determined for a few microorganisms generally yeilding little information applicable to identification. However, many of the studies have used an excitation wavelength near absorbance maxima. Some data indicates that excitation maxima of bacteria are distinct from absorbance maxima. Dalterio et. al. determined that emision spectra from widely different bacterial species appeared to be differential (Dalterio et. al., 1987).

3. Determination of excitation spectra at fixed emission wavelength. When excitation spectra were measured at an emission wavelength of 450 nm, Nelson et. al. were able to demonstrate definite differences between S. epidermidis, E. cloacae, and P. fluorescens. Another study reports broad excitation spectra (between 300 and 420 nm) of five species of bacteria representing five genera (Escherichia coli, Bacillus subtilis, S. epidermidis, E. cloacae, and P. fluorescens) when emission was measured at 430 nm. These excitation spectra appeared to be

differential for the five organisms studied and the differential quality was increased when the excitation spectra were determined at an emission wavelength of 455 nm.

4. Determination of combination excitation and emmission sectra (Shelly, Warner, & Quarles, 1980). As expected, this application of primary fluorescence yeilds much more information than either excitation or emission spectra alone. However, the time required to obtain these may be a limitation. Again, a high density of cells of pigmented pseudomonads (~8 x 10^6, Shelly, Warner, & Quarles, 1980) were required to obtain a sufficient signal.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. May not be sensitive enough for single cell detection. It should be noted that in all of the studies cited above, bacterial densities of 1 x 10^8 to 1 x 10^9 were used.

A. No addition of fluorochrome is necessary. These studies detect naturally fluorescing biomolecules, thus eliminating the need for the introduction of fluorochromes.

A/D. Depending on the requirement for obtaining recoverable detected microorganisms, the destructive potential of this method may be used to the advantage in killing any microbes that are been detected.

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6.0 Flow Cytometry

CONCEPT: Single Cell Detection

METHOD: Secondary Fluorescence, Mixed Dye Fluorimetry

DESCRIPTION: A sample of interest is incubated with a fluorescent dye for an appropriate period of time. Unbound dye is removed by centrifugation or filtration. The flourescence is then measured with a fluorimeter employing appropriate excitation wavelengths.

INSTRUMENTATION:

Fluorometer or flow cytometer with interface to computer.

Alternatively, for analysis of cells on a filter, a fluorescent microscope either configured for analysis by human observation or equipped with an automatically scanning mechanical stage, interface to a photodetector and computer, and possibly an image analyzer.

CHEMICAL PARAMETERS (ACTIVE SPECIES): The fluorescent dye employed confers the signal of interest.

SENSITIVITY: A single bacterial cell could be detected.

VOLUME: With concentration of microbes in a sample on a filter, the sensitivity of this method is essentially the same as epifluorescence.

SPECIFICITY: By employing fluorescent dyes that specifically stain DNA or protein this method can distinguish animate from inanimate objects. Variations of this method have been used to analyze mammalian cells, fungi, bacteria, and algae.

INVASIVE OR NON-INVASIVE: This method could be configured to either invasive or non-invasive sampling. Samples could be removed from the compartment of interest and concentrated by filtration through a membrane filter (pore size 0.2 or 0.1 um) either before or after staining. Alternatively, the mechanics of this method could be configured such as to continuously monitor a flowing stream as in the application of flow cytometry. The latter method has been employed for over a decade for the analysis of cell populations of both eukaryotic and prokarytic cells. However, the latter configuration would require the undesireable introduction of a fluorescent dye into the sample stream.

DESTRUCTIVE OR NON-DESTRUCTIVE: If appropriate fluorescent dyes are employed this method is non-destructive.

ID POTENTIAL: Low

MANIPULATION: Configured as an invasive technique, this method requires a number of manipulations of both solid and liquid media. Some of the objects, such as the filters, would require sensitive handling to prevent damage. All of the manipulations would require aseptic technique. To accomplish the manipulations by robotics would involve a relatively major effort in the development of the specific instrumentation. If the manipulations were performed by the crew, it is estimated that 0.5 man hours per sample would be required on earth to prepare the sample for microscopic analysis.

REVIEW OF APPLICATIONS AND VALIDITY: This method has primarily been applied to the analysis of populations of cells in pure culture. A signal can be measured in the order of microseconds thus making the method desirable for near-real time analysis.

LIMITATIONS(L), ADVANTAGES (A), & DISADVANTAGES (D):

L. If qualitative data is required this method cannot be used with samples that contain microorganisms that display multicellular patterns of growth.

A. Can employ dyes relatively specific for biomolecules such as DNA or protein.

A. If configured in the non-invasive manner, this method could provide for near-real time information regarding the microbial content of the sample.

D. The relatively involved sample manipulation, if configured for invasive sampling, is disadvantageous in that it requires either a significant dedication of crew time or a significant development in robotics. Experts in robotics should be consulted in order to determine feasabilty and cost regarding the employment of this technology.

D. If configured in the non-invasive manner, this method would require the introduction of undesired chemicals into the sample stream.

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7.0 Epifluorescence

CONCEPT: Single Cell Detection

METHOD: Secondary Fluorescence, Epifluorescence

DESCRIPTION: A sample of defined volume is removed from the compartment of interest and filtered through a filter (pore size TBD, at present 0.1 um is recommended). The retained material is stained with a fluorescent dye. At present acridine orange is the most commonly used dye, other dyes that have been used include 4',6-diamino-2-phenylindole, acriflavine, and bisbenzimide (Bergstrom, Heinanen, and Salonen, 1986). Residual stain is removed by washing the filter with buffer, and the filter is transferred to position for microbial analysis. The number of microorganisms are determined by assessing the number of fluorescent objects on the filter resembling microbes.

INSTRUMENTATION:

Fluorescent microscope (specs TBD) either configured for analysis by human observation or equipped with an automatically scanning mechanical stage, interface to a photodetector and computer, and possibly an image analyzer.

possible required fiber optics interfaces:

Image analysis processor

Spectrophotometer (fixed wavelength or scanning, TBD)
with interface to computer

CHEMICAL PARAMETERS (ACTIVE SPECIES): The fluorescent dye employed confers the signal of interest.

SENSITIVITY: The sensitivity of this method is essentially determined by the volume of sample that is practical to filter and the resolution of the optics employed. At present, with routinely employed techniques, a sensitivity of one microorganism ($\geq$ 0.2 um) per volume filtered is possible with human evaluation of the stained filter. This sensitivity could be increased to one microorganism of size $\geq$ 0.1 um per volume filtered by employing a 0.1 um pore size filter.

VOLUME: With the microbial specifications for the Space Station set at present at <1 bacterium/protozoan/fungus per 100 ml of potable water it is recommended that a minimum of 500 ml be sampled at each sampling time. It has been reported (Mittlerman, Geesey, & Hite, 1983) that a minimum of one liter of sample of ultrapure water was required in order to obtain the recommended number of cells per field for enumeration. It is possible that as much as 5 liters may be required (Mittleman, 1985).

SPECIFICITY: Particles other than microbes may bind the dye. A human can be trained to distinguish inanimate from animate

objects. At present training is required to teach the analyst to discriminate between cells and debris properly. For automated processing, image analysis will be required to distinguish animate from inanimate objects. There are reports of detection of bacteria, fungi, and protozoa with this method. By allowing formation of microcolonies on selective media before staining the filters and subsequent analysis it is possible to enumerate selective groups of bacteria such as coliforms, pseudomonads, and staphylococci (Rodrigues and Kroll, 1988).

INVASIVE OR NON-INVASIVE: This is an invasive technique requiring a relatively large sample size to confirm compliance with currently recommended standards.

DESTRUCTIVE OR NON-DESTRUCTIVE: This is a potentially destructive method. There is little information regarding the recoverability of microorganisms that have been processed by the procedure dictated by this method. The recommended (Pettipher, 1983) acidic wash (pH 3.0) and isopropanol rinse followed by drying will in all likelihood kill most microorganisms. In addition, the acridine orange may be cidal as well.

IDENTIFICATION POTENTIAL: Very low. (See CHEMICAL PARAMETERS)

MANIPULATION: This method requires a number of manipulations of both solid and liquid media. Some of the objects, such as the filters, would require sensitive handling to prevent damage. All of the manipulations would require aseptic technique. To accomplish the manipulations by robotics would involve a relatively major effort in the development of the specific instrumentation. If the manipulations were performed by the crew, it is estimated that 0.5 man hours per sample would be required on earth to prepare the sample for microscopic analysis. It has been reported (1) that quantitative results for the complete procedure can be available in three hours with appropriately trained personnel. An evaluation of the required personnel time in microgravity should be performed. All sample analysis after sample retrieval and staining could be automated with the development and verification of an appropriate self-focusing microscope, an automated scanning system, and a sufficient image analysis system. A semi-automated process has been evaluated (Pettipher, 1982)

REVIEW OF PUBLICATIONS; APPLICATIONS AND VALIDITY: An extensive review, discussion of the historical development, and description of the applications of the direct epifluorescent filter technique (DEFT) up to 1983 has been published (Pettipher, 1983). This technique has been used to detect bacteria in raw milk (Pettipher, 1983), viable but non-culturable Vibrio sp. and other bacteria from aquatic environments (Daley and Hobbie, 1975, Colwell et.al. 1985), viable phagocytized Yersinia pestis within macrophages (Straley and Harmon, 1984), viable and non-viable bacteria in ultrapure water (Mittleman, Geesey, and Hite, 1983, Mittleman, 1985), and bacteria in milk (Rodrigues, 1984), as well as many other applications. A method employing epifluorescence

is described as ASTM D4455 (Anon. 1985). Nuclepore (Pleasanton, CA) has a commercial kit available for routine laboratory and field determinations. Some reports in the literature (Pettipher, 1983) indicate a higher viable count when determined by this method compared to viable plate counts, however, application to evaluation of intravenous fluids (Denyer and Ward, 1983) indicated a close correlation between epifluorescent counts and pour plate counts. In the latter study both enumerating both green and orange-red fluorescing cells yielded the best correlation to the viable plate count indicating that cells determined to be dead by the acridine epifluorescent technique may not be dead. In fact, there is no agreement regarding the correlation between the color of fluorescence and viability (Pettipher, 1983). Because of the variability of staining reaction (color) as a function of procedure, the inconsistant results reported in the literature, and the possibility that not all enviromental microorganisms may behave like laboratory strains, the use of acridine orange epifluorescence for the determination of viable cells is questionable. However, it certainly has merit when used to enumerate total cells.

A recent modification of this technique has been reported where microcolonies were allowed to form before staining and epifluorescence evaluation (Rodrigues and Kroll, 1988). These investigators were able to obtain results within 3 to 6 hr at a contamination level above 10^3 per gram of a variety of foods for selective groups of coliforms, pseudomonads, and staphylococci.

By employing image analysis, this method has been used to detect and enumerate marine bacteria (Sieraki, Johnson, and Sieburth, 1985).

REQUIREMENTS FOR CALIBRATION AND INFLIGHT QUALITY CONTROL: In order to assure the validity of the method in-flight and to employ quality control, this method requires verification of:

1. The retainability of the filters employed.

2. The low background fluorescence of the filters employed.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. Not intrinsically specific for biological entities.

A. Applicable to bacteria, protozoa, and fungi.

A. Relatively long track record regarding reliability and comparison of epifluorescent counts with water quality.

D. Not applicable to viruses.

D. The relatively involved sample manipulation is disadvantageous in that it requires either a significant dedication of crew time or a significant development in robotics. Experts in robotics should be consulted in

order to determine feasabilty and cost regarding the employment of this technology.

D. Analysis of the dyed filters requires either the time of trained individuals or the employment of computer image analysis. Experts in image analysis should be consulted in order to determine feasabilty and cost regarding the employment of this technology.

D. Specific toxic hazards for acridine orange are not available. This chemical is designated as harmful if inhaled or swallowed (see accompanying Material Safety Data Sheet).

D. No evidence that the Aphragmabacteria are detected by this method.

D. No evidence that bacterial or fungal spores or protozoan cysts are detected by this method.

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8.0 Secondary Fluorescence -Use of Fluorophore Tag

CONCEPT: Single Cell Detection

METHOD: Secondary Fluorescence, Immunofluorescence

DESCRIPTION: The sample to be analyzed is mixed with an appropriate fluoresently labelled antibody. Unbound reagent is then removed by filtration or centrifugation. Fluorescently labelled microorganisms are then detected with a fluorescent microscope. Almost all the comments regarding mixed dye fluorimetry can be applied here with the following additional comments. A modification of epifluorescence could be applied, substituting specific fluorescent antisera for the more general staining fluorochromes employed in epifluorescence, to detect species and serotypes of designated interest.

INSTRUMENTATION:

Fluorescent microscope with interface to photodetector.

CHEMICAL PARAMETERS (ACTIVE SPECIES):

SENSITIVITY: A single cell could be detected.

VOLUME: See mixed dye fluorimetry.

SPECIFICITY: The employment of specific antibodies in this method makes it highly specific to the level of serotype identification with appropriate antibody. This high degree of specificity, however, detracts from an application where the diversity of microorganisms in a sample is unknown but where general enumeration is required. Polyspecific mixtures of antisera could circumvent this, however, it is impractical to raise antisera to all serotypes of all microorganisms, fluoresently label the immunoglobulins, and then mix the preparations in proportions that would achieve thew goal. This method is a highly desirable adjunct method to the microbial monitor for identification of microorganisms potentially isolated or for verification of identities potentially indicated by the data obtained from the monitor analysis. A modification of epifluorescence could be applied, substituting specific fluorescent antisera for the more general staining fluorochromes employed in epifluorescence, to detect species and serotypes of designated interest.

INVASIVE OR NON-INVASIVE: Most commonly invasive techniques are use to obtain samples for immunofluorescence.

DESTRUCTIVE OR NON-DESTRUCTIVE: This should allow for the recovery of viable cells provided the fluoresent antibody preparations are devoid of complement.

ID POTENTIAL: Very high.

MANIPULATION: See mixed dye fluorimetry.

REVIEW OF APPLICATIONS AND VALIDITY: Immunofluorscence has been employed in clinical, diagnostic, and research laboratories for many years to detect or verify species or serotypes of interest. A small sample of applications is included in the list of references for this method.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. A specific fluorescent antibody will detect only the corresponding serotypes.

A. The specificity afforded by this method allows for positive identification at the species or serotype level.

A. The specificity of this method with the employment of monospecific antisera makes positive detection of microbes after sample processing a relatively simple task for man or machine.

D. The relatively involved sample manipulation is disadvantageous in that it requires either a significant dedication of crew time or a significant development in robotics. Experts in robotics should be consulted in order to determine feasabilty and cost regarding the employment of this technology.

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9.0 Electrical Impedence - The Bactometer

CONCEPT: Single Cell Detection

METHOD: Electrical Impedence - The Bactometer

DESCRIPTION: When microorganisms grow and metabolize in a culture medium the products liberated change the composition of the medium. The changes in medium composition are accompanied by a change in impedance. In general, as microorganisms grow the impedence of the media decreases with time.

INSTRUMENTATION:

CHEMICAL PARAMETERS (ACTIVE SPECIES): the impedance of an inoculated sample is compared against a reference. Based on the time required to obtain a signal the density is calculated.

SENSITIVITY: $10^2 - 10^3$/mL

SAMPLE PREPARATION:

VOLUME: 2mL - 100mL

TIME REQUIREMENT

SPECIFICITY: Will detect those cells capable of growth. Technique may may be modified for specific groups.

INVASIVE OR NON-INVASIVE: Invasive

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-destructive

IDENTIFICATION POTENTIAL: Limited, however, may be modified to detect certain groups. ie coliforms, gram negative, gram positive, etc.

MANIPULATION: Little manipulation required other than sample collection.

REVIEW OF PUBLICATIONS; APPLICATIONS AND VALIDITY: Various workers have used impedance systems developed in their own laboratories for microbial studies. Ur and Brown [17] detected small inocula of several bacteria and a Mycoplasma species within 2 h, and demonstrated the inhibitory effect of antibiotics. Wheeler and Goldschmidt [18] quantified organisms from urine specimens by first filtering out leukocytes and debris in a coarse filter, washing the filtrate, and resuspending the microorganisns in distilled water. Using a nomograph, they related electrical measurements made in their instrument at an input frequency of 10 Hz to measurements made previously with known concentrations of microorganisms. Good correlation with standard laboratory methods was achieved, but the results were available in much shorter time periods. In another impedance

study, after routine blood culture broth bottles were inoculated with patient's blood, Kagan, et al. removed 30 ml of the broth and subjected it to a lysis-filtraiton procedure. The filter pad was put into a culture bottle connected by stainless steel electrodes to an empedance measuring device. In a study of 264 blood culture specimens, the impedance method detected 36% more positive cultures than a conventional broth method (49/53 compared eith 30/53). More fully automated refinements of this promising system that have been proposed have not yet been achieved.

Impedance measurements have several applications for the clinical microbiology laboratory, and in most instances, the results are available within short time periods. The Bactometer 32 system has been commercially available; however, no new applications or further development for its clinical use are in progress. The Bactometer 8 and 32 are limited by their small test capacity. For these instruments, uninterrupted on-line monitoring of the culture vessel is an advantage, but the work-load volume is restricted. The Bactometer 120 can handle 480 specimens in four incubators; however, for continous monitoring, dedicated incubators must be used with attachments that allow the electrodes to be plugged into the control unit. The prototype incubators were large and required an inordinate amount of laboratory space. Other systems that have been described depend on equipment not usually available in the clinical microbiology laboratory and also require the time and interest of experienced individuals to compile and "debug" the units.

REQUIREMENTS FOR CALIBRATION AND INFLIGHT QUALITY CONTROL:
Calibration and inflight QC relatively simple and reliable.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Several clinical laboratory applications

D. Same-day result

A. Frequent, continuous monitoring

L. Characteristics of microbial growth may delay detection

L. No Automated system available

D. Manual systems labor-intensive

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10.0 Electrical Impedence - The Coulter Counter

CONCEPT: Single Cell Detection

METHOD: Electronic particle counting

DESCRIPTION: Cells or particles in suspension are detected as they pass through an orifice across which is imposed a voltage potential.

INSTRUMENTATION:

Coulter Counter^{R}

CHEMICAL PARAMETERS (ACTIVE SPECIES): As particles pass through the orifice of the instrument changes in voltage are detected. These changes are proportional to the size of the particle detected.

SENSITIVITY: This method enumerates individual particles as they pass through the orifice.

VOLUME: This method could potentially be configured as an in-line detection method.

SPECIFICITY: Both inanimate and animate particles are enumerated.

INVASIVE OR NON-INVASIVE SAMPLING REQUIRED: This is a non-invasive technique

DESTRUCTIVE OR NON-DESTRUCTIVE: This is a non-destructive method.

ID POTENTIAL: No identification potential.

MANIPULATION: This method requires little or no manipulation of the sample before results are available.

REVIEW OF APPLICATIONS AND VALIDITY: Due to problems associated with fluid flow in microgravity this method may be of questionable value for space application.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

D. The orifice tends to clog frequently. This potentially could require considerable maintenance of the instrument and limit its development into a fully automated system.

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For additional references for electronic particle detection see previous method.

11.0 Scanning Electron Microscope

CONCEPT: Single Cell Detection

METHOD: Scanning Electron Microscopy

DESCRIPTION: A sample is passed through a filter, allowing for the entrapment of cells on the filter surface. After a subsequent processing, which includes glutaraldehyde fixation, drying and gold/palladium coating, the surface is examined by scanning electron microscopy.

INSTRUMENTATION:

Scanning electron microscope.

Support equipment for sample processing.

Possible image analyzer.

CHEMICAL PARAMETERS (ACTIVE SPECIES): No specific parameter. Morphological attributes of particles entraped on the filter indicate microorganisms.

SENSITIVITY: A single microbe can be detected. In published studies higher counts are generally achieved by this method when compared to epifluorescence (Balazs, 1987).

VOLUME:

As described for epifluorescence.

SPECIFICITY: Not very specific, however some species exhibit morphologies indicative of or unique to them.

INVASIVE OR NON-INVASIVE SAMPLING REQUIRED: This is an invasive technique

DESTRUCTIVE OR NON-DESTRUCTIVE: This technique is destructive due to the fixation and critical drying required and subsequent heavy metal coating.

ID POTENTIAL: Except for those microbes that exhibit genus or species specific morphologies, this method has little identification potential.

MANIPULATION: This method requires significant manipulation of the sample before results are available. In practice, individuals are generally higly trained in order that minimal artifacts are introduced during sample processing.

REVIEW OF APPLICATIONS AND VALIDITY:

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Yeilds higher counts than other techniques.

D. Specific training required.

D. Large weight, power and volume requirements.

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12.0 Limulus Amebocyte Lysate

CONCEPT: Biomolecular Detection

METHOD: Limulus Amebocyte Lysate

DESCRIPTION: The lysate of blood cells of the horseshoe crab (Limulus polyphemus) reacts with endotoxin and forms a clot.

SENSITIVITY: 10E-12 g of lipolysaccharide (LPS) is detectable. May be able to detect 10E-15g LPS. Since an average Gram-negative bacterium contains approximately 10E-14 g LPS, it may be possible to detect a single cell. However, it should be mentioned that the amount of LPS per bacterial cell is not constant from species to species and may vary as a function of environmental or growth conditions.

SAMPLE PREPARATION: With the current specifications a sample would have to be concentrated before analysis by this method.

TIME REQUIREMENTS: The assay can be performed in a relatively short time period (10 to 90 minutes).

VOLUME: A relatively large sample would have to be obtained and concentrated prior to performing this assay.

SPECIFICITY: Low reactivity, dependant on endotoxins-cell wall constitutents of gram negative cells.

ID POTENTIAL: Low

AD/DISADVANTAGES:

A. Standards readily obtainable for quality control.

L. Amount of endotoxin varies from cell to cell.

L. Specific for Gram-negative cell types.

D. Cell debris may interfere with determination of viable cells.

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13.0 Bioluminescence

CONCEPT: Biomolecular detection

METHOD: Bioluminescence

DESCRIPTION: Assay for ATP

INSTRUMENTATION:

CHEMICAL PARAMETERS (ACTIVE SPECIES):

SENSITIVITY: 10^3- 10^4 organisms/mL

SAMPLE PREPARATION: Fire fly luciferase preparations are made fresh daily. Enzyme reaction mixtures have to be prepared and samples added to 1mL this mixture. ATP soln serves as a standard.

TIME REQUIREMENTS: 1-2 hours, however, some researcher have detected 10 CFU/mL within 6-10 hrs.

VOLUME: Typically less than mililiter volumes are required.

SPECIFICITY: All organisms except viruses contain ATP. Reaction of ATP from any orgin will occur.

DESTRUCTIVE OR NON-DESTRUCTIVE:

INVASIVE OR NON-INVASIVE:

ID POTENTIAL: Low

AD/DISADVANTAGES:

D. Required detection limit not without modification.

D. Sample manipulation required.

D. Freshly prepared reagents required.

A. The technique is highly specific and the results are obtained rapidly within a few minutes or hours.

A. Only viable cells are determined.

A. The method offers a high degree of sensitivity, accuracy, and reproducitility

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14.0 Secondary Fluorescence - Addition of Fluorophore Enzyme Profiling

CONCEPT: Biomolecule detection

METHOD: Secondary Fluorescence - addition of flourophore Enzyme profiling

DESCRIPTION: The presence of an enzyme is detected by a change in the fluorescence of the bacteria-substrate mixture

SENSITIVITY: 10^4/mL

SAMPLE PREPARATION: Little or no sample preparation is required other than sample collection

TIME REQUIREMENTS: 2-8 hrs depending on initial concentration

VOLUME: Typically less than or equal to 1mL

SPECIFICITY: Specific for the target enzyme

ID POTENTIAL: High when a number of substances used with preculture isolute

AD/DISADVANTAGES: The advantages of enzyme tests over conventional ones have been discussed in detail. The advantages are related to the rationale of testing for individual enzyme activities, as opposed to testing for products of complete pathways (the basis of many conventional tests) or the detection of cell constituents (the basis of many chromatographic and serological techniques), and the increased sensitivity obtained by judicious choice of test conditions and use of sensitive equipment for measurement.

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15.0 Gas Chromatography

CONCEPT: Specific metabolic product detection

METHOD: Gas chromatography

DESCRIPTION: Detection of chemical compounds which are indicative of microbial contamination. FID, ECD and mass selective detectors may be used. A variety of target compounds may be identified and detected.

SENSITIVITY: Femtomoles at target compounds have been detected.

MANIPULATION: Sample manipulation is required.

SAMPLE PREPARATION: Generally some sample preparation is required in addition to collection.

TIME REQUIREMENTS: 1-2 hours

VOLUME: Large volumes 100 ml to 1 liter may be required.

SPECIFICITY: Certain groups of microorganisms may be identified however, for sensitivity generally detection.

ID POTENTIAL: low

LIMITATIONS (L), ADVANTAGES (A) AND DISADVANTAGES (D):

A. This method is sensitive enough for single cell detection.

A. Some information could be gained as to characterization based on certain phyisological groups.

D. This method is invasive and destructive.

D. This method has relative large instrumentation requirements for analyis and sample handling.

L. Relative large volume requirements.

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16.0 Raman Spectroscopy

CONCEPT: General Molecular Composition

METHOD: Raman Spectroscopy

DESCRIPTION: The sample is irradiated by monochromatic light. The resulting luminescent radiation (Raman Scattering) is detected. Maximum signal strength is obtained when the exciting wavelength is resonent with the absorbtion band.

INSTRUMENTATION: Raman Spectrometer consists of a laser light source (commonly argon) with frequency filtering and beam shaping optics, which produces a single frequency gaussian beam. This filtered beam is focused on the sample using an optical microscope.The Raman scattered light is then collected and directed to the detector a single frequency at a time using a monochromator arrangement. The most advanced instruments use a prism and photodiode array for light detection to gather a full spectrum in milliseconds rather than minutes.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Each molecule has a specific Raman luminescence pattern.

SENSITIVITY: Resonant Raman signals yeild high sensitivity. Acceptable Raman signatures for small numbers (<10) of highly pigmented bacteria and algae have been reported (Nelson et.al.) in the literature. By using a microflow cell or captive drop sample chamber (so that a majority of the sample is in the tightly focused beam) single cell detection may be possible, at least for some types.

VOLUME: Highest sensitivity is acheaved when the sample organism is confined to a small volume.

SPECIFICITY: Each molecule has a specific Raman spectra. Computerized pattern recognition and complete reference library would be necessary for identifying mixtures.

INVASIVE OR NON-INVASIVE If sampling can be done via a micro-flow cell or captive drop the method would be entirely non-invasive. When samples are taken by membrane filtration the method is of course invasive.

DESTRUCTIVE OR NON-DESTRUCTIVE: The methode may be destructive when high power UV lasers are used for exciter radiation.

ID POTENTIAL: It should be possible to identify any species that has been previously cataloged.

MANIPULATION: A flow cell would require no sample manipulation. The relatively simple manipulation involved in filration sampling might be suitable for automation.

REVIEW OF APPLICATIONS AND VALIDITY:

LIMITATIONS, ADVANTAGES, & DISADVANTAGES:

A. May be completely non-invasive and non-destructive with good ID potential.

A. High sensitivity

L. Single cell detection may be limited to highly pigmented bacteria.

D. Argon and excimer lasers require relatively large amounts of power.

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17.0 IR Spectroscopy

CONCEPT: General Molecular Composition

METHOD: IR Spectroscopy

DESCRIPTION: Cells are supported on an appropriate substrate, and IR radiation is absorbed by the sample.

INSTRUMENTATION: Fourier transform spectrometers are usually more sensitive than dispersion spectrometers. Beam focusing is required for small samples. As with most optical techniques, IR instrumentation is not particularly power intensive and may be amenable to automation/miniaturization.

CHEMICAL PARAMETERS (active species): Each type of molecule has a distinctive IR spectrum.

SENSITIVITY: High sensitivity, ROM picograms of isolated material. Sensitivity in aquious suspentions may be much lower. Raman spectroscopy is usually prefered for aquious samples.

VOLUME: Beam can be focused to 25-30 microns. Sample must be fixed in a small volume using sub-millimeter ID tubing or by drying the sample on an appropriate substrate (transparent for absorbtion and opaque for scattering measurements).

SPECIFICITY: Each molecule has a specific spectrum,thus the IR signatures of microorganisms are unique.

INVASIVE OR NON-INVASIVE: Microbes must be suspended in a small volume (non-invasive) or filtered and fixed on a substrate (invasive).

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-destructive when performed in situ. ,destructive when microbes are dried on a substrate, which is the usual method for aquious samples.

ID POTENTIAL: ID potential is very good, although computer supported pattern recognition would be required for mixtures. The accuracy of such ID's could be very good if all species present in the sample are contained in the spectrum library. It is imprtant to note that several researchers have reported that the IR signatures of microorganisms are affected by neutrients avalable in the growth media, as well as growth phase of the culture. The ID potential of this method may be "too good" since it may discern subtle differences in individuals of the same genus and species.

MANIPULATION No manipulation would be required for a capillary flow cell, but this may not be suitable for aquious substrates. Fixing cells on a matrix and drying is very labor intensive and technique critical.

REVIEW OF APPLICATIONS AND VALIDITY:

LIMITATIONS ADVANTAGES and DISADVANTAGES: IR has limited use for in situ aquious measurements, and sample preparation for substrate fixing/drying techniques is labor intensive, invasive, and destructive.

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18.0 GC/Mass Spectroscopy

CONCEPT: General Molecular Composition

METHOD: GC/Mass Spectroscopy

DESCRIPTION: Gas Chromatography coupled with mass spectroscopy (GC/MS) is a excellent analytical procedure for separation and characterization of certain organic compounds. Of course, the compounds of interest must be volatile or at least capable of derivitization to compounds of acceptable volatility. In most cases, a extraction step is required to isolate the target analyte in a concentration suitable for analytical detection.

INSTRUMENTATION: A sample concentrator, gas chromatograph, mass selective detector.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Bacterial cellular components, i.e., muramic acid, D-amino acids, diaminopimelic acid. In addition, certain compounds specific for specific bacterial groups may be targeted, i.e., heptose or rhamnose for Gram negatives, etc.

SENSITIVITY: Nanogram samples are required

VOLUME: Relatively large volumes of sample would have to be concentrated, probably through a filter, to achieve the required sensitivity.

SPECIFICITY: The specificity would be dependent upon the target molecule chosen, however there are a number of target compounds which would signify the presence of bacteria and/or fungi.

ID POTENTIAL: Identification is probably limited to specific physiological groups, i.e. bacteria, fungi, Gram negative, Gram positive, etc.

INVASIVE OR NON-INVASIVE: This technique is invasive. A sample must be collected and processed prior to anlysis.

DESTRUCTIVE OR NON-DESTRUCTIVE: This technique is destructive.

SAMPLE MANIPULATION: A sample would be collected through filtration of a large volume of water or air. The target compound would then be extracted and derivitized for analysis.

REQUIREMENTS FOR CALIBRATION AND INFLIGHT QUALITY CONTROL: Calibration and quality control could easily be accomplished in-flight.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. This method is sensitive enough for single cell detection.

A. Some information could be gained as to characterization based on certain phyisological groups.

D. This method is invasive and destructive.

D. This method has relative large instrumentation requirements for analyis and sample handling.

L. Relative large volume requirements.

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19.0 Mass Spectroscopy/Mass Spectroscopy

CONCEPT: General Molecular Composition

METHOD: "Hyphenated techniques" MS-MS,

DESCRIPTION: The problems of separation ,identification and quantitation of the analyte are the basic hurdles in any new analytical project. The state of the art in solving the most difficult of these problems are the so called "hyphenated techniques".These methods involve using high performance separation techniques with sensitive new detectors which yield several types of identification data.

INSTRUMENTATION: Combination of mass selective and ion trap detectors

CHEMICAL PARAMETERS (ACTIVE SPECIES): Molecular fragments. Fragmentation patterns differ according to the energy used to create the fragments. Common methods for this bond breaking are chemical ionization (CI), electron ionization (EI), and photoionization (PI).

SENSITIVITY: Nanogram samples are necessary.

VOLUME: Lyopholized samples or samples collected by filtering relatively large volumes would probably be necessary given the low contamination levels projected. Microliter volumes of concentrated aquous suspensions, or gasous "head-space" samples are a possibility.

SPECIFICITY: Each molecule has a specific fragmentation pattern. Since many types of molecules would be present in a microbial sample, separation should precede fragmentation.

ID POTENTIAL: Each microbial species has a specific chemical composition and will yield a specific "finger-print" fragmentation spectrum.

INVASIVE OR NON-INVASIVE: Usually invasive,with the exception of gasous head space analysis.

DESTRUCTIVE OR NON-DESTRUCTIVE: All of these methods completely destroy the sample, precluding culture or other identification techniques.

SAMPLE MANIPULATION: Sample manipulaion is similar to that required for pyrolysis. Filtration of a lerge volume of process water followed by collection and drying is labor intensive and technique critical.

LIMITATIONS, ADVANTAGES, & DISADVANTAGES:

A. These methods are sensitive enough for single cell detection.

D. They are invasive and destructive.

L. This state of the art technology has not been applied to micrboial identification as yet, and are unproven for this application.

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20.0 Visible Spectroscopy

CONCEPT: General Molecular Composition

METHOD: Visible Spectroscopy

DESCRIPTION: Monochromatic visible light is passed through the sample and the attenerated beam intensity is measured by a photodeitector.

INSTRUMENTATION: light source, monochrometer, photodetector, sample holder, PWR supply, photodiode instruments are faster.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Colored molecules i.e. molecular species which absorb viable light

SENSITIVITY: approximately 1 x 10^{-5} molar for very strong absorbers (i.e. hemoglobin)

VOLUME: Highest sensitivity is achieved when the sample organisms are confined to a small volume.

SPECIFICITY: Visible absorption bands are typically broad but unique. Separating mixtures would require computer and might be difficult since broad bands give little information.

INVASIVE OR NON-INVASIVE: Similiar to Raman

DESTRUCTIVE OR NON-DESTRUCTIVE: Similiar to Raman

IDENTIFICATION POTENTIAL: less than Raman - Bacteria must be highly pigmented. Broad bands have limited information.

MANIPULATION: Similiar to Raman

REQUIREMENTS FOR CALIBRATION AND INFLIGHT QUALITY CONTROL: relatively easy to calibrate - automatic

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Easily miniaturized

D. Limited I.D. potentials

D. Detection limits are unacceptable

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21.0 UV Spectroscopy

CONCEPT: General Molecular Detection

METHOD: UV Spectroscopy

DESCRIPTION: Ultraviolet light is passed through a sample and the attemuated beam intensity is measured by photodetector.

INSTRUMENTATION: Similiar to Visible

CHEMICAL PARAMETERS (ACTIVE SPECIES): Most organic molecules have a distinct UV spectrum especially proteins and nucleic acids.

SENSITIVITY: Depending on absorption of chemical species present ROM 10^{-5} - 10^{-8} Molar

VOLUME: Similiar to Visible

SPECIFICITY: Each molecule has a specific UV absorbance pattern.

INVASIVE OR NON-INVASIVE: May be non-invasive

DESTRUCTIVE OR NON-DESTRUCTIVE: Dependant upon wavelength used.

IDENTIFICATION POTENTIAL: Better than visible but not as good as Raman, UV spectra are not complicated. Resonance raman UV has good identification potential and may be more common in the future.

MANIPULATION: Similiar to Visible

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. Sensitivity

L. Chromaphores must be used to increase detection limits.

A. Some identification and/or discriminatory information is gained from UV spectra.

REFERENCES:

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22.0 Pyrolysis, Gas Chromatography

CONCEPT: General Molecular Composition

METHOD: Pyrolysis - Gas Chromatography

DESCRIPTION: Microorganisms are pyrolysed to yield biomolecule decomposition products, principly sugars, amino acids, and nucleic acids.These volitile decomosition products are then analized by gas chromatrography.

INSTRUMENTATION: Pyrolysis chamber followed by a gas chromatograph with FID, NPD or MSD as detector.

CHEMICAL PARAMETERS (ACTIVE SPECIES): The chromatograph of biomolecule decomposition products serves as a "fingerprint" for each microbial type according to its specific chemical composition. The relative abundance of certain amino acids is especially characteristic of a particular species.

SENSITIVITY: Not applicable to single cells.A large fraction of microbial decomposition products are not organism specific (H_2O, CO_2, CH_4, etc.) 200-800 microgram samples are required to produce nanogram (NPD) to picogram (MSD) quantities of each analyte for detection.

VOLUME: A significant volume must be millipore membrane filtered to produce a microbial sample of several hundred micrograms. If potable water supplies regularly achieved <1 CFU / 100 mls many liters would have to be filtered to provide a sample.

SPECIFICITY: Chromatograms of biomolecule decomposition products are specific to each type microbe, and differ for the chromatograms of debris decomposition products.

ID POTENTIAL: Chromatograms of biomolecule decomposition products are excellent "fingerprints" for identification. This method would require a library of chromatograms including all species encountered in or to make automated identification of mixtures.

INVASIVE OR NON-INVASIVE: The method is intensely invasive requiring filtration of large sample volumes.

DESTRUCTIVE OR NON-DESTRUCTIVE: Pyrolysis completely destroys the sample, precluding further analysis or culture.

SAMPLE MANIPULATION: A large sample must be membrane filtered. The fiter is removed from the filter chamber, and flash dried. The dried sample is placed in the pyrolysis chamber and the analysis is performed.

LIMITATIONS, ADVANTAGES, & DISADVANTAGES:

D. This method is invasive and destructive.

D. Single cell detection is improbable.

L. Real-time monitoring is not possible.

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23.0 Radioimmunoassay

CONCEPT: General Molecular Detection

METHOD: Radioassay

DESCRIPTION: Several methods involving the use of radioisotopes have been used for low level detection of particular biochemical species and bacterial growth.

INSTRUMENTATION: Dependant upon radioisotope used (generally alpha and beta particles.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Specific radioisotopes or radio-labled biochemicals.

SENSITIVITY: 10^3 cells per milliliter.

VOLUME: Small volume requirements but large volumes of sample can be concentrated on filters prior to analysis.

SPECIFICITY: High

INVASIVE OR NON-INVASIVE: Invasive

DESTRUCTIVE OR NON-DESTRUCTIVE: Usually non destructive

IDENTIFICATION POTENTIAL: Generally low but can be modified to specific physiological groups by incorporation of a growth step.

MANIPULATION: Some sample manipulation is required.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

L. Limited to specific physiological groups.

A. High sensitivity.

A. Can be coupled with immunospecific antibodies for highly specific identification potential.

D. Uses low level radiolabled compounds.

D. Instrumentation requirements.

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24.0 Volatile Product Detection/Membrane Filtration

CONCEPT: General molecular composition

METHOD: Dection of volatile cell components and analysis by gas mass spectroscopy.

DESCRIPTION: Cells are cconcentrated on membrane filter. Membrane filter is incubated on a nutrient soaked pad. Head space analysis conducted over time.

SENSITIVITY: Same as membrane filtration.

SAMPLE PREPARATION: None

TIME REQUIREMENTS: 2-6 hours

VOLUME: 100 mL

SPECIFICITY: Depends on media constituents

ID POTENTIAL: good

AD/DISADVANTAGES:

A. Uses standard instrumentation and methodology

A. Provides viable organisms for archiving or subsequent analysis

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Yabuuchi, E., Moss, C.W. (1982) Cellular fatty acid composition of strains of three species of Sphingo-bacterium gen. nov. and Cytophaga johnsonae. FEMS Microbiol Lett 13:87-91

Yabuuchi, E., Kaneko, T., Yano, I., Moss, C.W. Miyoshi, M. (1983) Sphingobacterium gen. nov., Sphingobacterium spiritivorum comb. nov., Sphingobacterium multiborum comb. nov. Sphingobacterium mizutae sp. nov., and Flavobacterium indologenes sp. nov., glucose-nonfermenting gram-negative rods in CDC groups IIk-2 and iib. Int Int J Syst Bacteriol 33:580-598

25.0 Thermal Photometry

CONCEPT: Single Cell Detection

METHOD: Thermal Photometry

DESCRIPTION: Variations in the physical properties of molecules occur as a result of their interaction with electromagnetic radiation. In thermal photometry interaction with light of specified wavelengths is monitored as a function of absorbtion of light energy of a second wavelength. The changes with respect to light interaction may be detected by observing changes in deflection, divergence, or diffraction of the incident laser beam.

INSTRUMENTATION:

Laser light source (two for some applications).

Photodetector with interface to computer.

Possible image analyzer.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Absorption of radiation by chemical compounds causes a change in refractive index which can be detected by laser beam probing. The volume detected can be very samll, in the range of nanoliters (1).

SENSITIVITY: To our knowledge, this methodology has not been applied to microbe or particle detection. It has been reported that 0.5 femtograms of azulene have been detected (1).

VOLUME: This methodology has been used in a through-sample detection configuration, thus no sample volume removal would be required.

SPECIFICITY: Unknown.

INVASIVE OR NON-INVASIVE SAMPLING REQUIRED: This is a non-invasive technique

DESTRUCTIVE OR NON-DESTRUCTIVE: It is not known whether this technique is destructive to living microorganisms.

ID POTENTIAL: Unknown, but probably low.

MANIPULATION: This method requires little or no manipulation of the sample before results are available.

REVIEW OF APPLICATIONS AND VALIDITY: With respect to application for microbial detection, this method is at best in experimental stages.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

REFERENCES:

1. Harris, J.M. 1986. Photothermal methods for detection of molecule liquids. Optics New. Oct. 1986. pp. 8-15.

26.0 Chemilulminescence

CONCEPT: Biomolecule detection

METHOD: Chemiluminescence

DESCRIPTION: the reaction of luminod with biologically active compounds, i.e. cytochrames, peroxidases, catalases, etc.

SENSITIVITY: 10^3 organisms/mL

SAMPLE PREPARATION: sample is reacted with luminal solution and the intensity of emitted light recorded and composed to standard

TIME REQUIREMENTS: preparation time plus 2 minutes.

VOLUME: 0.005 mL

SPECIFICITY: low

ID POTENTIAL: low

AD/DISADVANTAGES:

A. Volume requirements are low, thus allowing for a possibility of increased sensitivity.

D. Blood components or cell debris may interfere with the reaction.

REFERENCES:

Cormier, M. J.; Hercules, P. M.; Lee, J. "Chemiluminescence and Bioluminescence"; Plenum Press: New York, 1913.

Ewetz, R.; Strangert, K. Acta Pathol. Microbiol. Scand., Sect B, 1974, 82, 375.

Miller, C. A.; Vogelhut, P. O. Appl. Environ. Microbiol., 1978, 35, 813.

Neufeld, H. A.; Conklin, J.; Towner, R. D. Anal Biochem., 1965, 12, 303.

Oleniacz, W. S.; Pisano, M. A.; Rosenfeld, M. H.; Elgart, R. L. Environ. Sci. Technol., 1968, 2, 1030.

Vasileff, T.P.; Svarnas, E.; Neufeld, H. A.; Spero, L. Experimentia, 1974, 30, 20.

27.0 Microcalorimetry

CONCEPT: Single Cell Detection

METHOD: Microcalorimetry

DESCRIPTION: As microorganisms grow heat is produced. Specific thermal profiles produced by a particular organism may also provide the mechanism for identification.

INSTRUMENTATION: Microcalorimeter

CHEMICAL PARAMETERS (ACTIVE SPECIES): Heat produced from metabolic processes.

SENSITIVITY: 10^5 - 10^6 cells per milliliter.

VOLUME: Limited to small volumes

SPECIFICITY: High

INVASIVE OR NON-INVASIVE: Invasive

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-destructive

IDENTIFICATION POTENTIAL: Moderate

MANIPULATION: Sample manipulation is required.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

D. Instrumentation requirements

D. Low sensitivity

L. Requires growth phase

L. Moderate to long time requirements

REFERENCES:

Arhammer M., Mardh P-A., Ripa T., Andersson K-E. (1978) Microcalorimetric study of the effects of cephalexin and cephaloridin on Escherichia coli and Staphylococcus aureus. Acta Pathol Microbiol Scand [B] 86:59-65.

Beezer A.E., Bettelheim K.A., Al-Salihi S., Shaw E.J. (1978) The enumeration of bacteria in culture media and clinical specimens of urine by microcalorimetry. Science Tools 25:6-9.

Binford J.S., Binford L.S., Adler P. (1973) A semiautomated microcalorimetric method of antibiotic sensitivity testing. Am J Clin Pathol 59:86-94.

Forrest W.W. (1972) Microcalorimetry. In: Norris J. R., Ribbons D.W. (eds) Methods in microbiology 6B. Academic, London, pp 286-318.

Levin K. (1977) Calorimetry, a time-honored technique with a potential in analytical work and cellular biology. Clin Chem 23:929-937.

Russell W.J., Farling S.R., Blanchard G.C., Boling E.A. (1975) Interim review of microbial identification by microcalorimetry. In: Schlessinger D. (ed) Microbiology - 1975. American Society for Microbiology, Washington DC, pp 22-31.

28.0 Polymerase Chain Reaction

CONCEPT: General Molecular Composition

METHOD: Polymerase Chain Reaction (PCR)

DESCRIPTION: The polymerase chain reaction utilizes viral reverse transcriptase to amplify genetic material which might be present in small undetectable quantities. Once the target genetic material is amplified standard gene probe technology can be used.

INSTRUMENTATION: Dependant upon the specific detection system used.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Specific detection of genetic material.

SENSITIVITY: 10-100 cells per milliliter

VOLUME: Relative small volume requirements, larger volumes may could be concentrated using membrane filtration technology.

SPECIFICITY: High

INVASIVE OR NON-INVASIVE: Invasive

DESTRUCTIVE OR NON-DESTRUCTIVE: Destructive

IDENTIFICATION POTENTIAL: High

MANIPULATION: Sample manipulation is required.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Method is relatively sensitive.

A. Method does allow for the identification of contamining species.

L. Methodology is relatively novel and at present conducted in research laboratories.

L. Gene probes are required for all possible contamining species for identification.

D. No single gene sequence has been identified at present for all microorganisms.

REFERENCES:

Gyllensten, U. B. and Erlich, H. A. (1988) "Generation of Single Stranded DNA by the Polymerase Chain Reaction and its Application to Direct Sequencing of the HLA-DQA Locus," Proc. Natl. Acad. Sci. 85: 7652-7656.

Haff, L.A., and Mezei, L.M. (1989) "Measurement of PCR Amplification by Fluorescence," Amplifications 1: 8-10.

Innis, M.A., Myambo, K.B., Gelfend, D.H. and Brow, M.A.D. (1988) "DNA Sequencing with Thermus aquaticus DNA Polymerase and Direct Sequencing of Polymerase Chain Reaction-Amplified DNA," Proc. Natl. Acad. Sci. 85: 9436-9440.

Mullis, K.B. and Faloona, F.A. (1987) "Specific Synthesis of DNA in vitro via a Polymerase Catalyzed Chain Reaction," Meth. Enzym. 155: 335-350.

Murakawa, G.J., Zaia, J.A., Spallone, P.A., Stephens, D.A., Kaplan, B.E., Wallace, R.B. and Rossi, J.J. (1988) "Direct Detection of HIV-1 RNA from AIDS and ARC Patient Samples," DNA, 7: 287-295.

Saiki, R.K., Gelfend, D.H., Stoffel, S., Scharf, S.J. Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) "Primer Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," Science 239: 487-491.

29.0 Surface Acoustic Wave Detection

CONCEPT: Single Cell Detection

METHOD: Surface Acoustic Wave (SAW) Detection

DESCRIPTION: Surface acoustic wave detection is accomplished by monitoring changes in acoustic waves as particulates and chemical species bind to polymer coated detectors.

INSTRUMENTATION: Detectors and simple data recording and storage device.

CHEMICAL PARAMETERS (ACTIVE SPECIES): Cells or specific chemical species.

SENSITIVITY: High

VOLUME: Small to large volumes can be monitored.

SPECIFICITY: High

INVASIVE OR NON-INVASIVE: Invasive but can be in-line and accomplish total volume monitoring.

DESTRUCTIVE OR NON-DESTRUCTIVE: Non-Destructive

IDENTIFICATION POTENTIAL: Low but with multiple detectors using specific coating materials can be significantly increased.

MANIPULATION: Sample manipulation is not required.

LIMITATIONS (L), ADVANTAGES (A), & DISADVANTAGES (D):

A. Method is relatively sensitive.

A. Method could allow for the identification of contamining species.

A. Instrumentation and detectors are simple and have no moving parts.

L. Methodology is relatively novel and at present conducted in research laboratories.

REFERENCES:

Murray, R.W., R.E. Dessy, W.R. Heineman, J. Janata, and W. R. Seitz, Eds., "Chemical Sensors and Microinstrumentation,"ACS Symposium Series 403, 1989.

Grate, J.W., A. Snow, D. S. Ballantine, Jr., H. Wohltjen, M. H. Abraham, R. A. McGill, and P. Sasson, Anal. Chem., **60**, 869, (1988).

Bein, T., K. Brown, G. C. Frye, and C. J. Brinker, J. Am. Chem. Soc., 111, 7640 (1989).

Harris, J.M., R. A. McGill, and M. S. Paley, Macromolecules, submitted.

Herren, B.J., S. G. Shafer, J. M. Van Alstine, J. M. Harris, and R. S. Snyder, J. Colloid Interface Sci., 51, 46-55 (1987).

Harris, J.M. and K. Yoshinaga, J. Bioact. Comp. Polym., 4, 281-295 (1989).

APPENDIX B

MANUFACTURERS

2.2.1 Laser Light Scattering

2.2.1.1 Laser Nephelometer

Sensitivity of 10^5 in 6 hours.

Instrument used manufactured by the Hoeschst Behring Institute. Neon laser tuned to 632.8 nm. Forward scattering measured at between 5 and 12^o.

2.2.1.2 DAWN Model B or Model F (batch or flow)

Laser light scattering

Wyatt Technology Corporation
820 E. Haley St.
Santa Barbara, CA 93103
(805) 963-5904

Model B ~$15,000
Model F ~$20,000

2.2.1.3 Climet Model CI-1000 Liquidborne Particle Counter

Laser light scattering

Available model ::

Model CI-1000 with CI-1010 sampler	~$14,000
CI-175-600 Sensor	~$ 3,000
	$17,000

J B Systems
C/O Climet Instruments Company
P O Box 2405
LaGrange, GA 30241

2.2.1.4 Unizak Model ERC 9320

can count particles down to 0.3 to 0.05 um

They claim they were the first to manufacture a high flow rate laser **airborne particle** counter with a sensitivity below 0.2 um.

J B Systems
C/O Climet Instruments Company
P O Box 2405
LaGrange, GA 30241

2.2.1.5 Light scattering polarization

Coherent Radiation
Palo Alto, CA — 3W argon laser at 488 nm

Spectra Physics
San Jose, CA — 5mW neon laser

Beckton Dickinson
Sun Valley, CA — for FACS argon

measures forward light scattering between 2 and 17°.orthogonal light scattering at between 115 and 65°.

2.2.1.6 Spectrex Corporation
3594 Haven Avenue
Redwood City, CA 94063
(415) 365-6567

Laser Light Scattering at 90°

Sub-micron particle detection (0.11 - 0.7 um)

Approximate cost: $ 30,000.00

2.2.1.7 TSI Clean Room Monitoring

TSI, Incorporated
P.O. Box 64394
St. Paul, MN 55164

2.2.2 Electonic Particle Counting

2.2.2.1 The Bactometer.

Vitek Industrial
595 Anglum Dr.
Hazelwood, MO 63402

Determines bacterial densitites by electrical impedence changes.

More than 24 hours required to detect 100 cfu / 100 ml.

2.2.2.2 Coulter Counter

Coulter Electronics, Inc.
590 West 20th Street
Hialeah, FL 33010

2.2.2.3 Bactobridge

T & M, Centronic Sales
King Henry's Drive
New Addition, Croydan CR90BE
England, UK

2.2.2.4 Bactomatic

A Division of Medical Technology Corp.
719 Alexander Road
P.O. Box 3103
Princeton, NJ 08540

2.2.2.5 Honeywell, Inc.

Gerald J. Wade Mail # 108
4800 E. Dry Creek Road
Littletown, CO 80122

2.2.2.6 Japan Tectron Instrument Corporation

Organization 6

2.2.2.7 Mathus System

Mathus Instruments Ltd. Site 1
Almandbonk, Perthshire
PH13NQ, UK

2.2.2.8 Goldschmidt and Wheeler

University of Texas

2.2.3 Flow Cytometry

2.2.3.1 Fluorescent activated cell sorter

Becton Dickinson FACS Systems Division
490-B Lakeside Drive
Sunnyvale, CA 94086

2.2.3.2 Laser Flow cytometer

Coulter Electronics, Inc.
590 West 20th Street
Hialeah, FL 33010

2.2.3.3 Laser Flow Cytometer

Kratel GmbH & Co KG
Boeblinger Strasse 23
D-7250 Leonberger-Stuttgart
West Germany

2.2.3.4 Mercury Arc Flow Cytometer

Ernst Leitz Wetzlar GmbH
D-6330 Wetzlar
West Germany

2.2.3.5 Cytofluorograph Flow Cytometer

Ortho Diagnostics Systems
Raritan, New Jersey 08869

2.2.3.6 Interactive Laser Cytometer

Meridian Instruments, Inc.

2.2.4 Fluorescence (including epifluorescence)

2.2.4.1 Nuclepore Epicount

Epifluorescence detection of microorganisms

2.2.4.2 Bactoscan

Fluorescent microscopy to enumerate bacteria in milk and urine -- sensitivity ~10^4/mL.

Foss, Denmark

2.2.4.3 Analytical Measuring Systems Ltd.
Shirchill, Saffron Waldon, Essex, UK

2.2.4.5 Photon Technology International
601 Ewing Street, Suite R-2
Princeton, NJ 08540

2.2.5 Other

2.2.5.1 Vitek Bioburden Card

Vitek Industrial
595 Anglum Dr.
Hazelwood, MO 63402

2.2.5.2 Spectrum III

Ortho Diagnostics Systems
Raritan, New Jersey 08869

2.2.5.3 Electro Chemical

2.2.6 The following is a list of Manufacturers/Distributors reporting subject systems.

2.2.6.1 Bacteriological Detection Systems

Analytical Measuring Systems
API Systems SA
Baxter Healthcare Corp. Miscroscan Div.
Becton Dickinson Diagnostic Instrument Systems
Becton Dickinson Microbiology Systems
Becton Dickinson Vacutainer Systems
BIOCOM
Costar Corp.
Croft Scientific Ltd.
Curtin Matheson Scientific, Inc.
Eldan Bio-Technologies (EBT) Ltd.
Fisher Scientific
Hybritech Europe S.A.
Los Alamos Diagnostics
Mast Laboratories Ltd.
Omega Diagnostics Ltd.
Organon Teknika (USA)
Ortho Diagnostic Systems Inc.
Radiometer America
Roche Diagnostic Systems
Tzafon M.L.P. Ltd.
WWR Scientific

2.2.6.2 Biological Monitoring Systems

BIOCOM
Curtin Matheson Scientific, Inc.
Fisher Scientific
Los Alamos Diagnostics
PBI International
Rush Enterprises
Sterilization Technical Services, Inc.
Sunquest Information Systems
VWR Scientific

2.2.6.3 Bacteriological Counters

Analytical Measuring Systems
Cambridge Instruments
Curtin Matheson Scientific, Inc.
Fisher Scientific
Paul Marienfeld KG
VWR Scientific

2.2.6.4 Microbiological Identification Systems

Access Medical Systems, Inc.
Analytab Products (API)
Analytical Measuring Systems
API Systems SA
Austin Biological Labs
Baxter Healthcare Corp, Microscan Div.
Beckman Instruments, Inc.
Beckman Instruments, Inc./Diagnostic Systems
Becton Dickinson Diagnostic Instrument Systems
Becton Dickinson Microbiology Systems
Behringwerke AG
Buhlmann Laboratories AG
CLONATEC-BIOSOFT
Costar Corp.
Curtin Matheson Scientific, Inc.
Diagnostics Pasteur
Du Pont Diagnostics
ECO-BIO N.V.
Eldan Bio-Technologies (EBT) Ltd.
Environmental Diagnostics, Inc.
Fisher Scientific
Flow Laboratories
Flow Laboratories (INT.) SA
Hewlett Packard Ltd.
F. Hoffman-La Roche & Co. AG
Mast Laboratories Ltd.
Medical Wire & Equipment Co. (Bath) Ltd.
Mercia Diagnostics Ltd.
Omega Diagnostics Ltd.
Organon Teknika (USA)
Organon Teknika
Pro-Lab, Inc.
Radiometer America
Roche Diagnostic Systems
Sensititre (UK) Ltd.
Vitek Systems
VWR Scientific

APPENDIX C

KC-135 DOCUMENTATION

Request for Reduced Gravity Test Support

REQUEST FOR REDUCED GRAVITY TEST SUPPORT
for
The Johnson Research Center
The University of Alabama in Huntsville
specifically
Real-time Microbiological Monitoring
under Low g Conditions

Brian L. Benson
Flight Investigator

Melvin V. Kilgore
RTMM Project Director

a. Test Objectives

* Verify that the test bed design successfully performs the necessary fluid handling tasks in low gravity. Circulation, Mixing, Innoculation, & Sampling

* Assess the Performance of a simple candidate monitor (particle counter) under aircraft operation and low g conditions.

b. Desired Schedule

* June 1989 - Manually operated test "shakedown run" with polystyrene calibration spheres.

* Aug 1989 - Comparative testing of simple monitor using *E. coli* culture and limited automation.

* Sept 1989 - Additional comparative testing possibly involving *Lactobacillus sp.* and increased automation.

c. Brief description of the test and associated test equipment

Background

This work will produce and test a closed loop microbiological test bed. Such a system is a necessary step in the Johnson Research Center's program to define and develop a near-real-time microbiological monitor (RTMM) to assure quality of water supplies aboard Space Station Freedom. In order to assess the performance accuracy and precision of candidate monitor technologies we must compare these automated electronic instruments with accepted conventional microbiological techniques. Simultaneous sampling of a simple "mock-up" closed loop recovery system which has been innoculated with a known concentration of microbes or particles is the best way to accomplish such comparative testing.

The ultimate goal of our program is production of a flight qualified microbiological monitor system for testing on the space shuttle. It is important that our designs incorporate the appropriate fluid handling techniques for a microgravity environment. The candidate electronic monitoring instruments may perform differently under low g conditions due to differences in fluid flow and mixing. Our designs must be hardened for flight

operations where conditions are much less forgiving than in the laboratory. KC-135 operations of the closed loop test bed will provide the design information and comparative testing essential to our continued progress.

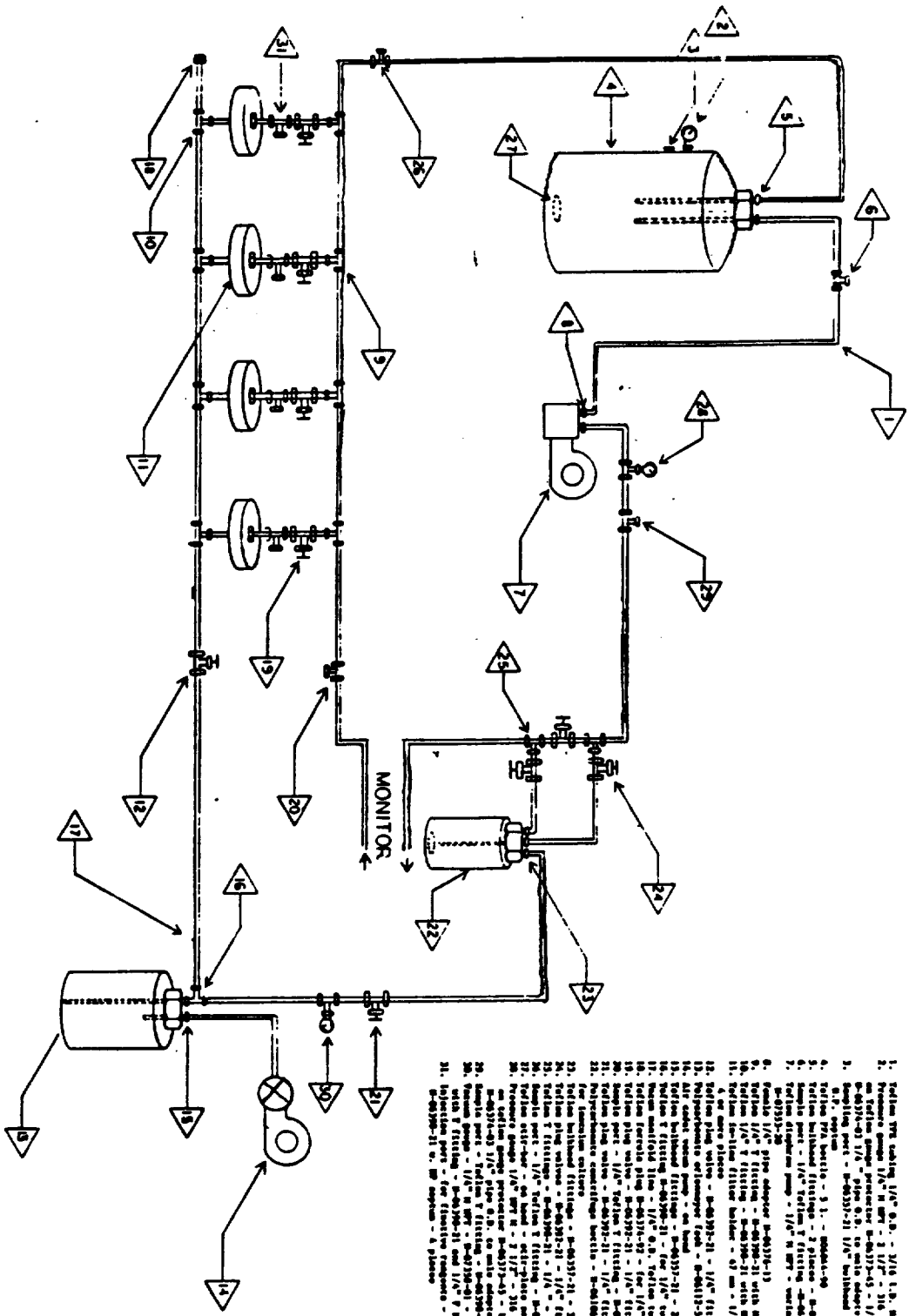
Test Equipment

The microbiological test bed (see fig. 1) consists of resevoirs (22,4,13), recycle loop (1), filters (11), pumps (7,14), and associated valves and monitoring equipment. All wetted surfaces are teflon TFE or high-density polypropylene. When pump motors and monitors are disconnected, the entire assembly is autoclavable. Total fluid capacity is approximately 10 liters. All fluid handling equipment will be double contained to prevent spills and the associated hazards.

Concept Test Protocol

During testing an innoculum solution of known concentration (in #22 of fig 1) is introduced into the recycle loop and resevoir (1&4) by means of valves (#24). This simulated contamination is then simultaneously monitored by a candidate electronic technology and conventional techniques via filters (#11) and sample ports (#20,etc).

FIGURE 1



CLOSED LOOP MICROBIOLOGICAL TEST BED (MTB)
PRELIMINARY SCHEMATIC

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d. Number of test personnel required for flight and a description of the requirement for each individual's presence

Three people are required to perform testing on the first flight series, with any two of the three required on subsequent flights. During the initial test Brian Benson will keep a time-line checklist and take close-up photographs of system operation. Muncell McPhillips will operate the monitor and Melvin Kilgore will take conventional samples. Subsequent flights would not require photographic documentation of hardware operation, and check list duties would be shared by two operators. Mr. Benson has flown Electrodeposition Program experiments for the UAH Consortium for Materials Development in Space on five occasions. Ms. McPhillips and Mr. Kilgore are currently scheduling the required physicals and physiological training for flight certification.

e. List special support required or constraints, including security classification of project, if applicable

none

f. Preliminary hazard analysis identifying generic hazards and controls

* Mechanical - Structural analysis is being performed, fluid system will be fully inclosed to prevent spills, sharp edges will be eliminated by design.

* Electrical - All electrical equipment will be properly grounded, protected from fluid contact, and fused or circuitbreaker protected on at least three levels.

* Biological Hazards - The initial flight series, which will be an equipment test, will not include any live bacteria; instead a sterile suspension of polystyrene micro-spheres in water will serve as a contamination model. In subsequent tests, a laboratory strain of **E. coli** and or **Lactobacillus sp.** may be used. The strains used are normally nonpathogenic and should present no hazard to healthy adults. **E. coli** is a normal resident of the human intestinal tract, and **Lactobacillus sp.** are commonly used in the food industry.

* Liquid Containment - Final flight design of the fluid handling equipment will include a waterproof enclosure to contain fluids and microbes. The enclosure will also separate fluid handling equipment from electrical equipment (pumps, monitor electronics, etc.)

g. Names, addresses, and phone numbers of contacts

Brian Benson
The University of Alabama in Huntsville
Johnson Research Center SB - 358
Huntsville, Ala. 35899
(205) 895 - 6391

Melvin Kilgore
The University of Alabama in Huntsville
Johnson Research Center CSC - 213
Huntsville, Ala. 35899
(205) 539 - 7620

The University
Of Alabama
In Huntsville

Consortium for the Space Life Sciences

SPMC Clinical Science Center
201 Governors Drive
Huntsville, Alabama 35801
Phone: (205) 539-7620

TEST EQUIPMENT DATA PACKAGE

for

The Consortium for Space Life Science's
Real Time Microbial Monitoring Program

specifically

Evaluation of Candidate Microbial Monitor
Technologies by Comparison with Conventional
Filter Techniques Under Low G Conditions

Brian Benson

Mr. Brian Benson
Flight Investigator

M V Kilgore

Mr. Melvin Kilgore
CSLS Laboratory Director

An Affirmative Action / Equal Opportunity Institution

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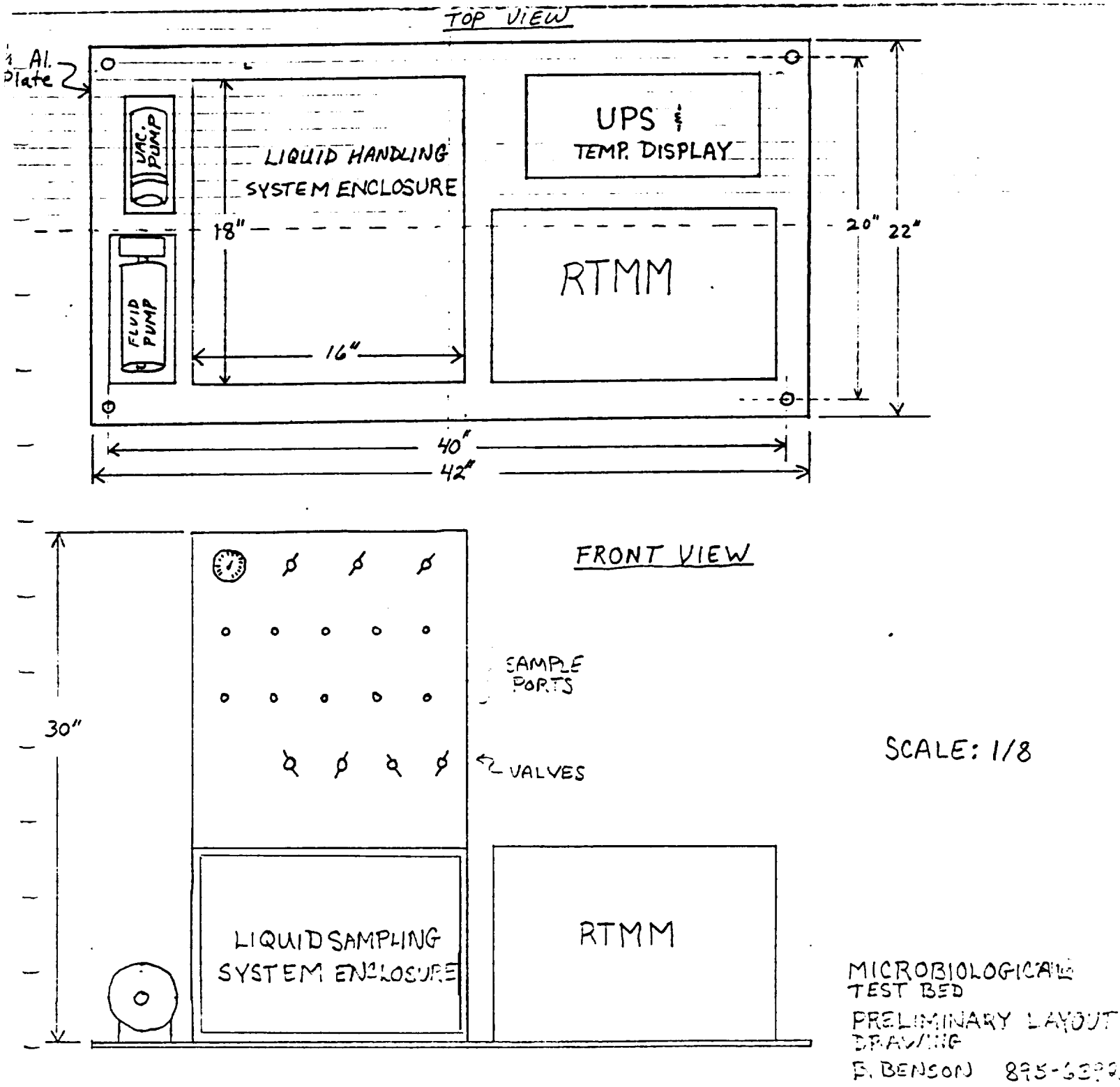
a. Synopsis

For more than a year the UAH Consortium for Space Life Sciences has been evaluating state of the art technologies for near-real-time microbiological monitoring of air and water supplies on the Space Station Freedom. Five techniques (based on light scattering and/or fluorescence) have been selected for field evaluation based on intensive literature review.

Recently, a microbiological test bed (MTB) (figure 1) was constructed to evaluate the performance of candidate monitor technologies in bench tests and in microgravity. The candidate real-time techniques will be compared to conventional filter samples acquired simultaneously. Low gravity (g) operations are essential since; (1) fluids handling equipment must perform correctly in low g, (2) differences in fluid behavior from 1 g to low g may cause real-time instruments with "flow cells" calibrated in 1 g to be completely wrong in low g, and (3) there is considerable evidence to suggest that bacteria grown in the spacecraft environment have substantially different surface morphology than their 1 g ancestors. The MTB and candidate monitor may some day provide a valuable research tool for investigating the effects of microgravity on bacterial growth, antibiotic sensitivity, and other physiological parameters of bacteria.

Figure 1 MTB Layout Drawing



b. Test Objectives

The objective of the proposed test is to evaluate the performance of a candidate real-time microbial monitor by comparing results obtained with the monitor with results obtained by slower standard methods. This would be accomplished using bench tests, KC-135 flights, and eventually the STS.

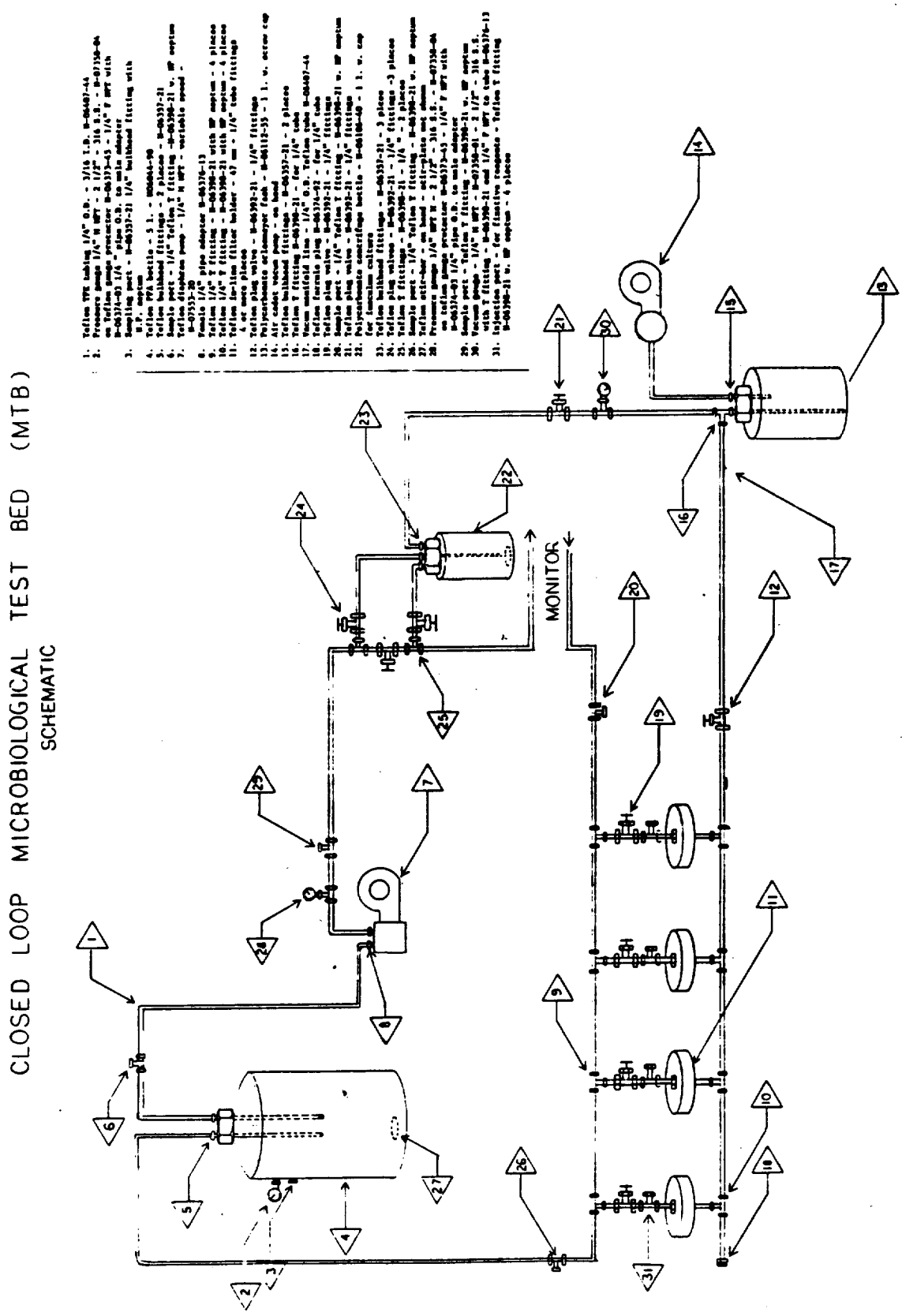
c. Test Description

The Microbiological Test Bed (MTB) is a closed loop recirculating system that includes the candidate microbial monitor and a series of filtration ports. The MTB is a simplest case model of a space station water recovery subsystem, where water is pumped from a storage vessel, physically or chemically processed in some manner, and returned to some storage vessel. In our simplest case the pretreatment and postreatment storage is in the same 10 l. high density polypropylene jar, and the only processing will be the controlled introduction of a known amount of "contamination", in this case polystyrene microspheres commonly used for calibration purposes.

These tests will be an evaluation of particle size/concentration profiles of a suspension of polystyrene microspheres as they are circulated through the test bed system. Samples of 100 ml. will be filtered under microgravity conditions using a system of valves (figure 2) . The filters will be removed and analyzed on the ground by florescence microscopy. This data will be compared to that obtained by the real-time monitor candidate (in this case a Met-One brand particle counter/sizer). The fluid handling apparatus will be visually monitored during the tests to identify obvious functional design defects such as:

(1) Bubble Formation - Bubble formation may cause some types of monitor to give false readings. The production and

Figure 2 Flow Diagram



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transport of small bubbles in microgravity may differ significantly from bench tests. Although this factor was considered carefully during design, only actual low g operation will reveal the true impact of bubble formation on the validity of the monitor data produced.

(2) Non-Homogeneity of the Circulating Solution - Gravity levels affect fluid flow. This may result in non-homogeneous suspensions of particles over time. Since the MTB has the capability of obtaining multiple samples from different locations simultaneously, the impact of this potential problem can be evaluated during low g operation.

(3) Dead Volume - The MTB was designed to minimize dead volume (areas where particles can remain trapped and not be monitored). However there are a number of places where dead volumes unavoidably exist. These volumes could result in the formation of nonrepresentative compartments that could lead to sampling errors. The direct syringe sampling ports allow access directly to the flowing stream. Samples collected trough these ports may be compared with filter samples and monitor data to evaluate the effect of dead volumes.

All fluid handling equipment in the MTB is Teflon PFE or high density polypropylene so that this system is biologically inert, autoclavable, and has a minimum of dissimilar materials.

CASE 1: The tensile on 2 JSC furnished fastners would recieve the worst load during a 9 g forward event. A load F_{1T} can be expected on each of two JSC furnished fastners.

M_1 = (156 lbs.)(9 g's)(15 in.) =21,060 in. lbs.

F_{1T} = [(21,060 in. lbs.) / (20 in)] (1/2) = 527 lbs.

* The ultimate tensile strength of each JSC furnished aircraft bolt is 5000 lbs.

M.S. = (5000 / 527) - 1 = 8.5

Analysis of Shear : If a Poisson's ratio of 1/2 is assumed -

Ultimate shear strength of each bolt is ... 5000/2 = 2500 lbs.

* Worst case loading would occur assuming entire weight is on the plane of the base plate

F_S = (156 lbs.)(9 g)/ 4 bolts = 351 lbs. per bolt

Marigin of Saftey = (2500 / 351) - 1 = 6.1

CASE 2: The tensile load toword the rear of the aircraft would be the worst load during a 9 g forword event. A load F_{2T} can be expected on 4 of the #10-32 bolts.

M_2 = (58 lbs.)(9 g)(15 in.) = 7830 in. lbs.

F_{2T} = [(7830 lbs) / (9 in.)](1/4) = 218 lbs.

The ultimate tensile strength of the S.S. #10-32 bolts is 2000 lbs. according to the manufacturer.

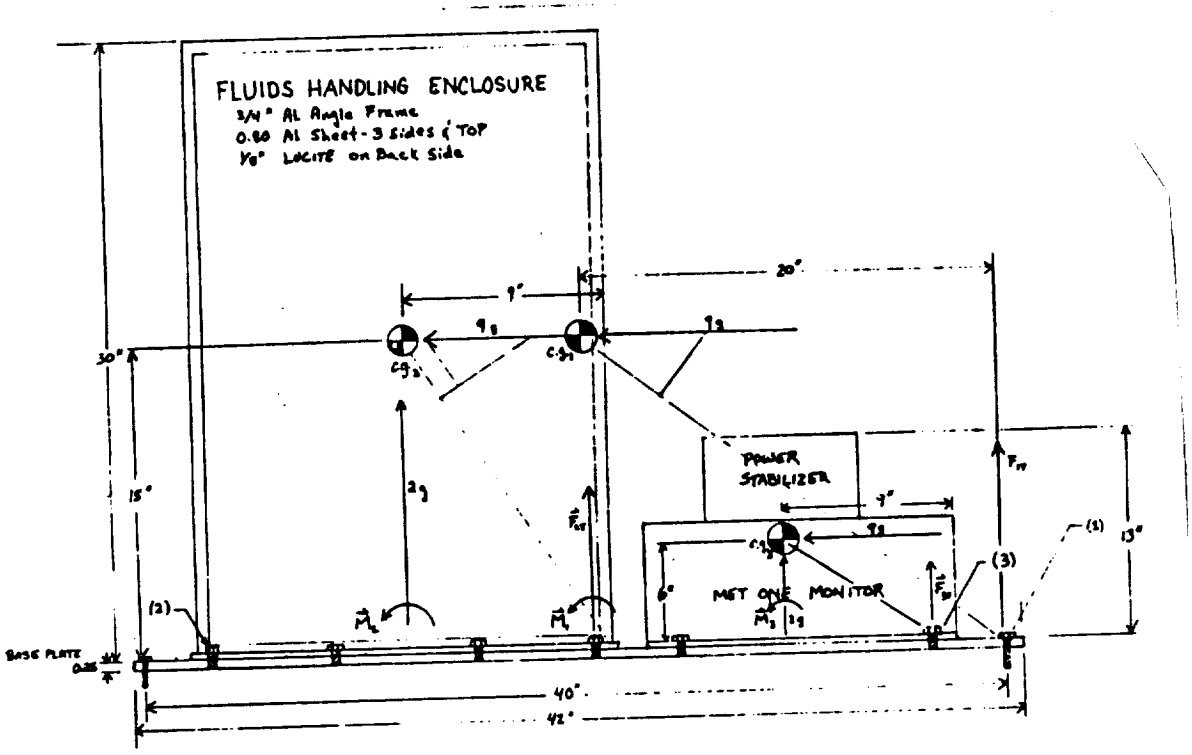
This gives a marigin of ... 2000 / 218 -1 = 8.2

This analysis is conservative since it neglects 4 additional bolts located along the sides perpendicular to the side of greatest stress.

d. Structural Load Analysis

Microbiological Test Bed

Assume c.g.'s at the geometric center of each component. The actual c.g.'s are lower since heavier components are located below the centerline. This analysis is of a case worse than the actual. the Fluids Handling Enclosure weighs 58 lbs when this system is filled with water, the monitor/power stabilizer stack weighs 63 lbs.. The entire assembly including pumps and base plate weights 156 lbs.



Worst case loading will be 9 g's forward and 2 g's up.

Three areas are analysed as possible failure points.

(1) Base plate to aircraft i.e. 4 JSC furnished 5000 lb. yield
(2) Fluid Handling Enclosure to base plate i.e. 16 #10-32 S.S. socket head bolts with helicoils
(3) Monitor/Power Stabilizer Stack to base plate i.e. 4 #10-32 socket head bolts with helicoils

For the 2 g up load -

F_{2T} = (58 lbs)(2 g) / 16 bolts = 7.3 lbs. per bolt

This is many times less than the 2000 lb. tensile rating.

Analysis of Shear - Worst case loading would be the entire 58 lbs in shearing in the plane of the bolt pattern.

F_S = (58 lbs.)(9 g) / 16 bolts = 33 lbs. per bolt

Far less than the shear rating of 1050

CASE 3: Tensile stress for a 9 g forward event on 2 of the four #10-32 S.S. bolts

M_3 = (63 lbs)(9 g)(6 in.) = 3402 in. lbs.

F_{3T} = [(3402 in. lbs.) / (9 in.)] (1/2) = 189 lbs per bolt

M.S. = [(2000) / (189)] - 1 = 9.6

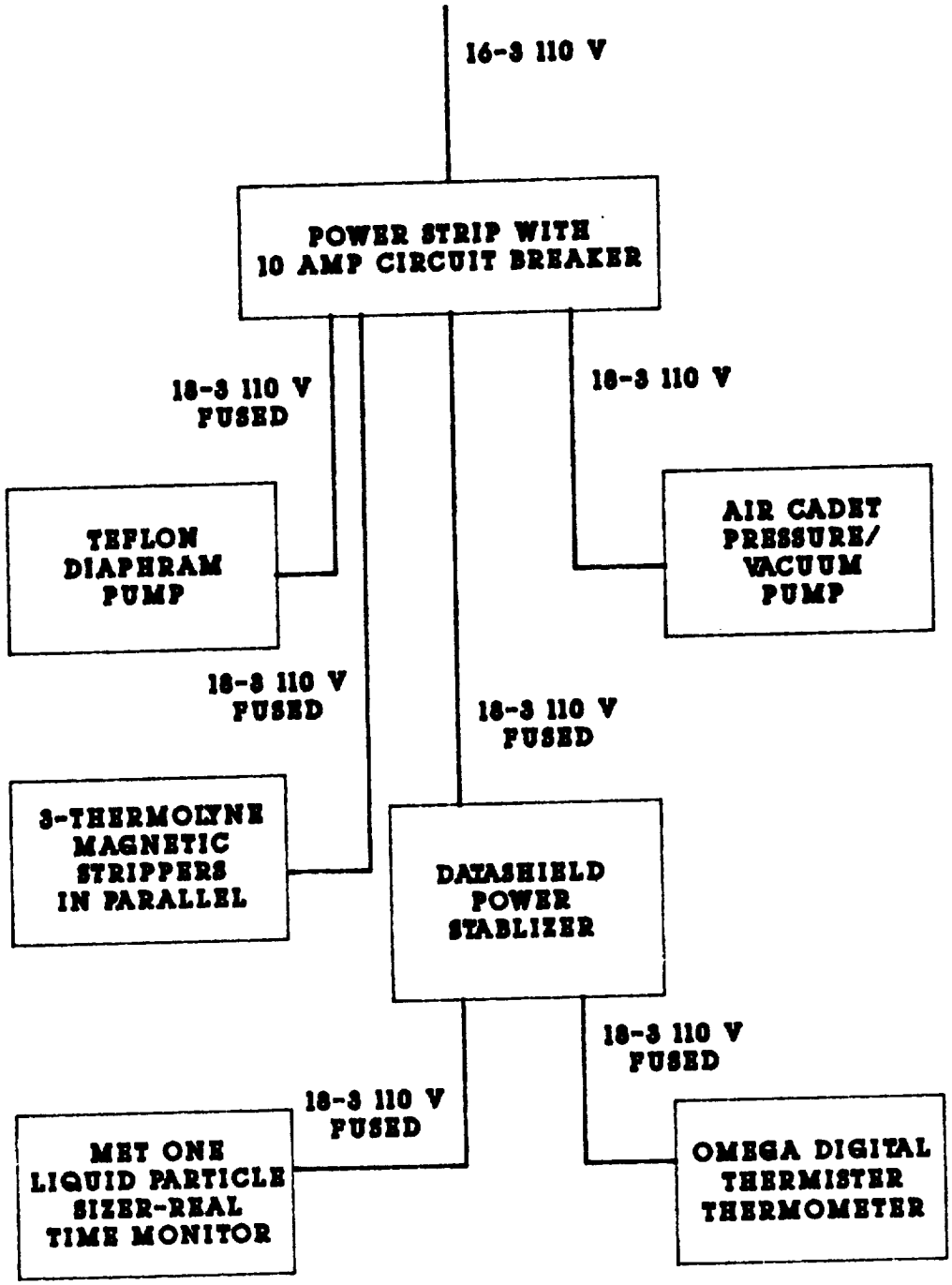
Analysis for the 2 g up case and pure shear case follow CASE 2

e. Electrical Load Analysis

The MTB uses only 110 Volt, 60 cycle for its operation. The total 110 VAC current draw is 7.35 amps. A Block diagram of the electrical system is given in figure 3.

110 VAC 60 Hz Equipment	
Cole-Palmer Teflon Diaphram Pump & Drive Model # 07090-42 & 07554-20	2.0 Amp
Air Cadet Vacuum/Pressure Pump Model # 7530-40	1.5 Amp
MET ONE (tm) Liquid Particle Counter Model # 210	1.0 Amp
Datashield (tm) Uninteruptable Power Supply Model # PC 200	2.0 Amp
Omega "Digicator" (tm) Thermistor Thermometer Model # 412B	< 0.1 Amp
Thermolyne Magnetic Stirrers - 3 @ 0.25 A Model # S17415	0.75 Amp
TOTAL 110 VAC POWER REQUIRED	7.35 Amp

Figure 3 Electrical Load Diagram



f. Pressure Vessel Certification

The MTB does not use any high pressure vessels requiring certification per section 5.2 of JSC-22803.

The MTB is a pumped closed-loop recycle system and may exhibit low pressure transients in the fluid flow lines, therefore gauges are installed to alert experimenters to any abnormal pressure trends. The diaphram pump is rated at 75 psig max.,the tubing and connectors are rated at 125 psig.

g. In-Flight Test Procedures

In-flight tests will involve taking syringe samples, conventional filter samples, and recording real-time data with the candidate monitor. The monitors are flow through instruments and will only require command entry via the keyboard during flight. Conventional filter samples are taken simply by opening and closing a series of valves. Syringe samples will be taken by inserting the needle of a large syringe (50 cc.) through a rubber septum port, withdrawing the appropriate amount of sample, and immediately capping the syringe and storing same in an appropriate rigid restrained container.

The required "checklist" type specific procedure is currently being developed in conjunction with bench testing, and will be submitted for review prior to the July 19 FRR.

h. Parabola Requirements, Number, and Sequencing

Only 6 to 8 parabolas per flight will be necessary. MTB operations may be accomplished regardless of flight profile.

i. Test Support Requirements, Ground and Flight

This experiment requires no supplies provided by JSC.

j. Data Acquisition System

No data acquisition connections are required to JSC equipment.

k. Test Operating Limits or Restrictions

There are no test limits or restrictions for this equipment.

1. Proposed Manifest

Three people are necessary to perform the tests. Mr. Brian Benson has flown on several flight series previously and would handle the syringe sampling. Mr. Melvin Kilgore would operate the valve system to take conventional filter samples. Dr. Muncell McPhillips would operate the Met One real-time monitor. No JSC personnel would be required.

m. Photographic Requirements

There are no photographic requirements for this experiment.

n. Hazard Analysis

(1) Mechanical - structural analysis is included in this document, all sharp edges are eliminated or padded.

(2) Electrical - All electrical equipment is properly grounded and fused or circuit breaker protected on at least two levels.

(3) Toxicity - There are no toxic materials used in this experiment.

(4) Fluid Handling - All fluid handling plumbing is isolated in the fluid handling enclosure to provide secondary containment for the 12 liters of water that fill the closed loop system. This secondary containment and good construction practices will preclude fluid spills and the associated mess and electrical hazard.

A complete hazard analysis is provided by MSFC Safety, Huntsville, Al. 35812.

Hazard Report Number One

Hazard Title: Fluid Pressure System - The pumped cloosed-loop fluid handling system may exhibit low pressure transients especially if valves are incorrectly manipulated.

Discription of Hazard: Possible rupture of fluid lines, connections, and associated fluid loss.

Hazard Cause: Incorrect manipulation of valves which direct flow or line blockage due to any cause.

Hazard Control:

1. Pressure gauges are installed to allert experimentors of any pressure increase so that corrective action may be taken before problems develope.
2. Fluid handling system is inclosed to prevent loss of fluid into the aircraft interior.
3. The diaphram pump is rated at 75 psi. max. pressure and tubing and connections are rated at 125 psi.

Verification Method:

1. System operation test witnessed by MSFC Saftey
2. Checklist operating procedure should prevent incorrect valve manipulation.

Verification Status:

1. Groundbased test according to checlist proceedure scheduled with MSFC Saftey for the week of 7-24-89.

Hazard Report Number Two

Hazard Title: Ignition Sources

Discription of Hazard: Possible ignition of electrical insulation or other materials due to electrical short circuit.

Hazard Cause: Electrical short circuit.

Hazard Control:

1. Electrical system is fused or circuit breaker protected on several levels and wiring is proper gauge for application.
2. Fluids are contained and isolated as much as possible from the electrical system.

Verification Method:

1. Electrical load analysis included in section e.
2. System has been inspected by MSFC Saftey

Verification Status: Analysis and inspection completed.

Hazard Report Number Three

Hazard Title: Electrical Shock.

Description of Hazard: Electrical shock to experimentors coming in contact with live 110 Volt power.

Hazard Cause: Improper construction or short circuit.

Hazard Control:

1. Electrical system is fused or circuit breaker protected on several levels and wiring is proper gauge for application.
2. Fluids are contained and isolated as much as possible from the electrical system.
3. System is contructed according to standard practices with no unprotected connections.

Verification Method:

1. Electrical load analysis included in section e.
2. System has been inspected by MSFC Saftey

Verification Status: Analysis and inspection completed.

Hazard Report Number Four

Hazard Title: Structural Failure

Description of Hazard: Loss of structural integrity during crash loads resulting in prjctile hazards to crew.

Hazard Cause: Improper design and/or construction.

Hazard Control: Proper design and good construction practices.

Verification Method:

1. Structural Load Analysis is included in section d.
2. Structure inspected by MSFC Saftey.

Verification Status: Analysis and inspection complete.

Hazard Report Number Five

Hazard Title: Mechanical

Description of Hazard: Injury to crew due to sudden aircaft maneuvers or turbulence.

Hazard Cause: Sharp edges and protrusions.

Hazard Control: Sharp edges eliminated by design or padded.

Verification Method: Inspection by MSFC Saftey.

Verification Status: Inspection complete.

The University Of Alabama In Huntsville
Huntsville, Alabama 35899
Telephone: (205) 895-6361

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MICROBIOLOGICAL TEST BED
PRELIMINARY LAYOUT DRAWING
E. BENSON 895-6392

Kenneth E. Johnson Research Center

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In Huntsville

Kenneth E. Johnson
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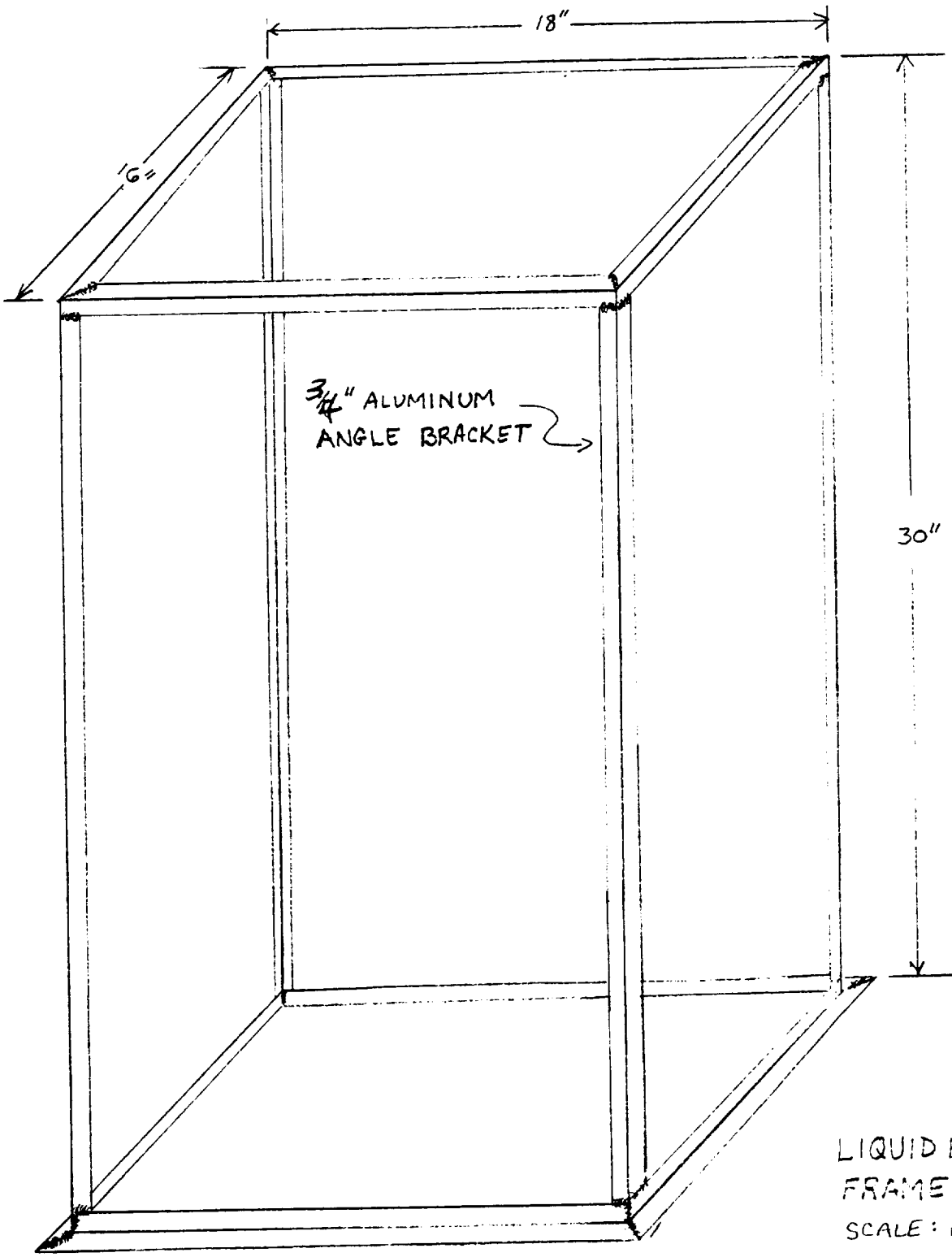

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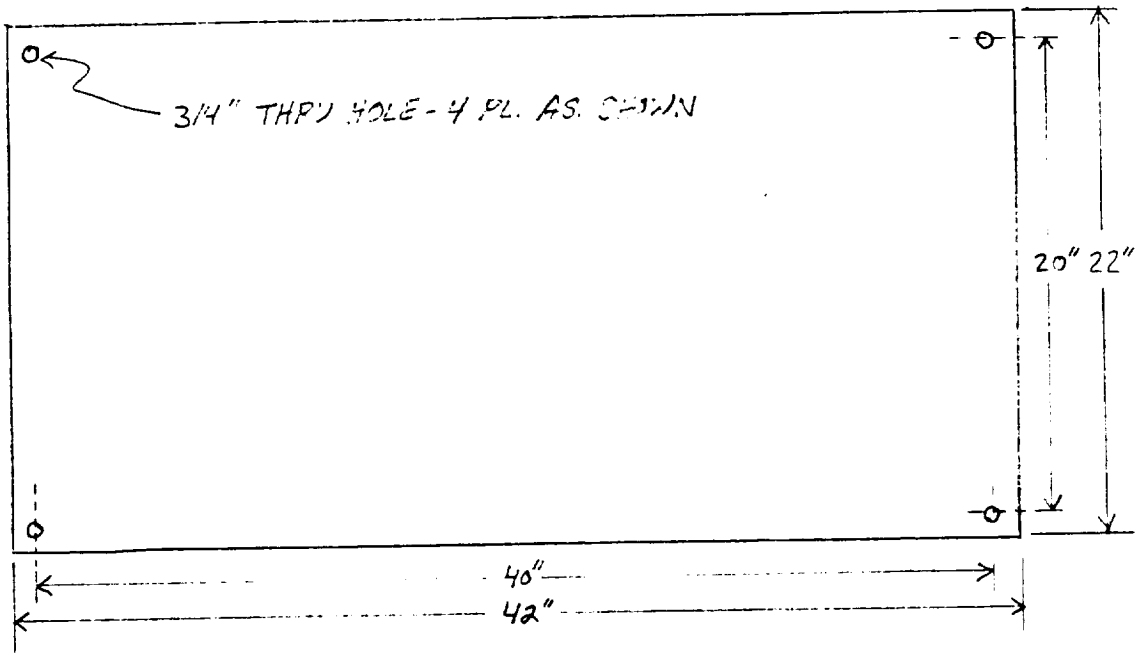

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APPENDIX D

PRESENTATIONS AND PUBLICATIONS

DEFINITION OF A MICROBIOLOGICAL MONITOR FOR APPLICATION IN SPACE VEHICLES

Melvin V. Kilgore, Jr.

Kenneth E. Johnson Research Center

and

Robert J. Zahorchak, Ph. D.

Department of Biological Sciences

Consortium for the Space Life Sciences

The University of Alabama in Huntsville
Huntsville, Alabama 35899

Contract No. 9-17963
Johnson Space Center
Duane Pierson, Ph. D.

EXPERIMENT OBJECTIVES:

PHASE I

- **Identify and Evaluate current methodologies for microbial monitoring**
- **Determine the Feasibility of Developing the Hardware for Space Applications**
- **Develop a Method for the Application of Microbiological Monitoring in Space**
- **Develop a Conceptual Design and Functional Diagram**
- **Prepare a Cost Estimate Regarding the Development Phase**
- **Define the Experimental Parameters to be Evaluated on Future STS Missions**

PHASE II

- **Thorough Evaluation of the Candidate Methodologies**
- **Development of Prototype Hardware**
- **Extensive Ground Based Evaluation of Hardware and Methodology**
- **In Flight Experiments**

Outreach Program

DEFINITION OF A MICROBIOLOGICAL MONITOR FOR APPLICATION IN SPACE VEHICLES

UAH

BACKGROUND

- **Necccessity for Microbiology Monitoring**
  Closed System Environment
  Increased Duration Missions
  Increased Distances
  Potential for Immuno Compromised Crew
  Experiments and Hardware

- **Current Methodologies**
  Particulate Detection
  Culture Techniques
  Indicator Organisms

- **Unique Requirements**
  Microgravity Conditions
  Multiple Sample Handling
  Power, Weight, Volume
  Analysis Time

- **Specifications**
  Water
  Air
  Surfaces

TECHNOLOGY NEED

- **No Commonly used Near Real Time Monitor Currently Available**

JUSTIFICATION

- **Assurance of Performance**

- **Bacterial Physiology Significantly Different in Space**

Outreach Program

DEFINITION OF A MICROBIOLOGICAL MONITOR FOR APPLICATION IN SPACE VEHICLES

UAH

EXPERIMENT DESCRIPTION:

PHASE I

- **Definition and Design of a Near Real-Time Microbiological Monitor for Space Applications**

PHASE II

- **Development and Evaluation of Performance of a Microbiological Monitor Under Microgravity and Other Conditions Imposed by Space**

CRITERIA FOR FLIGHT EXPERIMENT

- **Should Provide Information Required for the Development of a RTMM**
- **Should Demonstrate Proof of Concept Under Microgravity Conditions**
- **Should be Self Contained and Require Little Crew Support**
- **Experimental Design Should be such that Results/Products can be Analyzed/Retrieved on the Ground**

TECHNICAL APPROACH

METHOD EVALUATION AND TRADE STUDIES

- **Technical**
  - Primary
    - Sensitivity
    - Time
    - Maturity
    - Applications
  - Secondary
- **Engineering**
  - Power
  - Weight
  - Volume
  - Expendables
- **Feasibility**
  - Precision
  - Compatibility
  - Complexity
  - Development
- **Cost**

EXTENSVIE GROUND BASED EVALUATION OF METHODOLOGY

DEVELOPMENT OF PROTOTYPE AND GROUND BASED STUDIES

PROOF OF CONCEPT (IN FLIGHT)

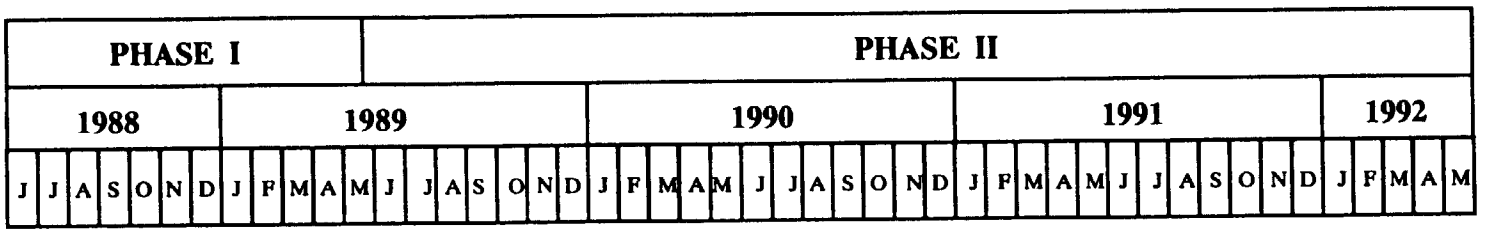
EVALUATION OF HARDWARE (IN FLIGHT)

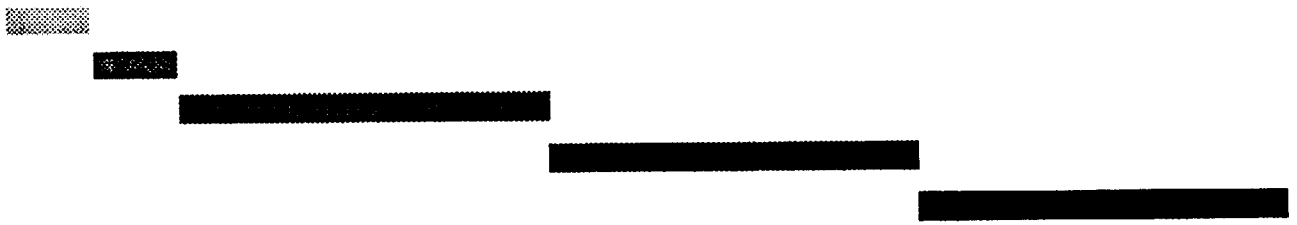
Outreach Program

DEFINITION OF A MICROBIOLOGICAL MONITOR FOR APPLICATION IN SPACE VEHICLES

UAH

SCHEDULE





REVIEW

DESIGN

EVALUATION

DEFINITION

DEVELOPMENT

FLIGHT EXPERIMENT

CHARACTERISICS Of A NEAR REAL TIME MICROBIOLOGICAL MONITOR

- **It Should be Adaptable to Water, Air and Surfaces**
- **It Should be Reliable and Require Little Maintenance**
- **If Should be Rapid**
- **I t Should be Self-Contained and Require Minimum Crew Support**
- **It Should provide for Crew and Ground Support Interactions**
- **It Should Lend itself to Improvements and Modifications toward both Quantitative and Qualitative Monitor**
- **It Should be ready for Incorporation Aboard SS Freedom**

Outreach Program

DEFINITION OF A MICROBIOLOGICAL MONITOR FOR APPLICATION IN SPACE VEHICLES

UAH

SUMMARY OF RESULTS

- **Identified Approximately 30 Methodologies having Potential Application to Microbiological Monitoring**
- **Approximately One-third of these met the Primary Requirements**
- **Five Highest Candidates from Secondary Screening choosen for Further Evaluation**
- **Engineering Trade Studies Currently Underway**
- **Feasability Studies Currently Underway**
- **Conceptual Design and Functional Diagrams**

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Definition of a Near Real-Time Microbiological Monitor for Application in Space Vehicles

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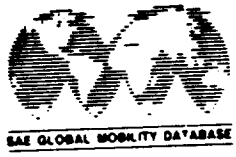

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ABSTRACT

The environment of Space Station Freedom will present unique challenges with respect to microbiological monitoring. Recycling of air and water will provide potential reservoirs and transmission routes for microorganisms. Since S.S. Freedom will operate as a marginally closed environmental system, sources of potential microbial hazard must be rapidly identified in order to minimize risk to the crew and structural or operational systems.

Our recent efforts have been directed toward the identification of relevant concepts and methodologies for microbiological monitoring in Space and to define the requirements of a near real-time microbiological monitor. Initially, five concepts applicable to microbiological monitoring were defined. Within these concepts, twenty-eight methodologies were identified which have the potential for microbial detection and/or identification. Evaluation criteria were developed and all identified methodologies were evaluated. Of these, only one-third met the minimum requirements that were established for a near real-time microbiological monitor. Through further technical evaluations, the five candidates yielding the highest scores were chosen for engineering and feasibility trades. The candidate methodologies include: laser light scattering, primary fluorescence, secondary fluorescence, volatile product detection and electronic particle detection. In addition, the advantages and disadvantages of the candidate method are described.

REGARDLESS OF THE OPERATING procedures used and the sophistication of the systems and subsystems onboard Space Station Freedom, microbial contamination of the Freedom environment is inevitable. Even if S.S. Freedom could be initially sterilized, crew members will continually shed microorganisms during normal activity and during crew and supply replenishment. Once introduced, microorganisms will proliferate in environments which provide the necessary nutrients and physical conditions. The reproduction of some bacterial species is very rapid and that of some fungi yield spores which can be readily disseminated. Thus, microorganisms, particularly bacteria and fungi, have the potential to become a significant problem onboard Freedom.

The consequence of unchecked bacterial proliferation on Freedom may result in health risks to the crew, damage to hardware or interference with system performance. However, the presence of microorganisms in the Freedom internal environment does not necessarily pose a health problem provided that 1) their numbers are controlled, 2) the proper environmental conditions are maintained, 3) the health of the crew remains stable, 4) the numbers of communicable pathogenic microorganisms are minimal, and 5) water, air and surfaces are microbiologically acceptable. In view of these concerns, methods will be employed to monitor microbial populations onboard S. S. Freedom.

The source of most of these organisms will be the crew. Because much of the microbial population will be derived from humans, the presence of at least opportunistically pathogenic organisms is likely. Even though crews can be screened and quarantined prior to their missions, intra-crew microbial exchange will occur. In addition, inter-crew microbial exchange may occur during crew rotations and may produce greater problems.

PAST EXPERIENCE

Microbiological monitoring of crew members began during the Gemini program (1)*. Pre- and postflight comparisons indicated an increase in the number of microorganisms, microbial simplification and microbial transfer between crew members occurred (2). During the early Apollo missions, illnesses were not uncommon; potentially pathogenic microorganisms were recovered postflight from crew members and intra-crew transfer of pathogens was implicated (1,3,4). During the longer duration Skylab missions, intra-crew transfer of pathogens was observed. Evidence was also obtained to support the theory of microbial

*Numbers in parentheses designate references at end of paper.

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simplification of anaerobes (5,6). Microbial build-up on surfaces and in the cabin air was demonstrated during these longer duration missions and appeared to be related to mission duration. Although Shuttle missions are of relatively short duration, air and surface contamination and microbial transfer between crew members have been reported (7).

The majority of the microbial flora encountered by crew members will be controlled by each individual's own host defense mechanisms. However, evidence also suggests that the immunological response of crew members may be compromised during space flight (8-10). In addition, experiments have indicated that certain bacteria may have increased resistance to antibiotics when grown under microgravity conditions (11). Other data is available which indicate that antibiotic treatment during space flight has not been optimally effective (12).

Microbial contamination of products manufactured during an STS mission has already been reported (13, 14). Regardless of the specific problems that may be encountered, unwanted microbial contamination of experimental apparatus, manufactured products or animals onboard the Freedom Station is not acceptable. The impact of life science experiments upon the microbial flora of the Freedom environment when using animals, plants, insects or microorganisms to meet experimental objectives must also be considered.

MICROBIOLOGICAL MONITORING

In order to assure the safety of the crew and to prevent possible microbial damage to certain components of the Freedom Station, the microbiological content of water and air supplies, as well as certain defined surfaces, must be assessed periodically. The failure to identify and eliminate microbiological hazards as they arise may result in serious risk to the crew or equipment onboard Freedom.

On Earth, one major identified concern for public health is that potable water supplies remain free of potential human pathogens. This concern is justified because many pathogens are transmitted from individual to individual through the fecal-oral route where water serves as a major reservoir. Considerable effort is used in monitoring water supplies on Earth for microbiological content.

The sampling, monitoring and evaluation of microbial parameters onboard Freedom presents several unique problems. First, the sample matrix is diverse, e.g., gas, liquid and solid. This suggests that different methods for sample collection and handling may be required. A desirable alternative would be to develop a method for microbiological monitoring that is applicable to all sample matrices of interest. Since the microbiological quality of water is a primary concern it is logical that the basic technology for microbial monitoring be designed for liquid samples. All samples could be easily transferred to a liquid medium as liquids are conveniently analyzed by conventional microbiological methods. Other design considerations include size, weight, power requirements, analysis time and conditions, reagents, manipulation of samples, and degree of crew involvement.

CURRENT REQUIREMENTS

The current proposed specifications regarding microbiological quality of the Freedom environment dictate that extremely sensitive methods for evaluation be employed (Table 1). At the present time, it is expected that in-flight analysis of both potable and hygiene water will be required prior to use. A minimum of 48 hours for potable water and 24 hours for hygiene water will be required before release of the water for use, during which time microbiological determinations will be conducted (15). The specified hold times are dictated by application of standard methods for microbial enumeration. In addition, periodic in-flight grab samples obtained from the water treatment systems will be conducted on a routine basis (15).

Air and surface samples will be collected periodically and analyzed for microbial content (1). Crew members, food products and experimental animals will be sampled on a contingency basis only (1). Likewise, microbial identifications (bacteria and fungi), as well as antibiotic sensitivity determinations, will be required on a contingency basis (1).

TABLE 1 TENTATIVE SPACE STATION FREEDOM MICROBIOLOGICAL SPECIFICATIONS (16)

PARAMETER	WATER (CFU/100mL)	AIR (CFU/m^3)	SURFACES (CFU/25cm^2)
BACTERIA			
TOTAL	<1	<1,000	<100
ANAEROBES	<1	—	—
AEROBES	<1	—	—
GRAM POSITIVE	<1	—	—
E. coli	<1	—	—
TOTAL ENTERICS	<1	—	—
YEAST AND MOLDS	<1	<1,000	<100
TOTAL MICROORGANISMS	<1	<1,000	<100

CURRENT METHODOLOGY FOR ANALYSIS

At the present time, the only available standard methods which meet current specifications are cultural. Current requirements dictate the use of cultural methods, since they are based on colony forming units (CFUs). Cultural methods do provide a technology for direct determination of certain microorganisms. The sensitivity of the method employed is based on the volume of sample to be analyzed. Drawbacks to the use of cultural methods are primarily that they are both labor and time intensive. In addition, they involve the promotion of microbial growth within the Freedom environment, thus generating undesirable biological waste and increasing the overall microbial population.

In support of these methods, they 1) have a proven track record on Earth, 2) should be readily adaptable to the Freedom environment, 3) are readily adaptable for detection of various physiological groups of microorganisms, and 4) meet the current water quality specifications and standards for testing. However, it is possible that the time required for conventional analysis of these parameters

in longer duration missions, including the production of potable and hygiene water in-flight, might prove to be unacceptable. The primary concerns in this situation are assuring the health of the crew and the reliability and proper operation of the environmental control and life support system (ECLSS).

Certain recent advances in the assessment of microbiological quality on Earth may have application to the Freedom environment. A likely candidate for initial microbiological monitoring is a direct epi-fluorescence filter technique (16). This method can be completed in 2-3 hours on Earth, and thus provides rapid determination of total microbial content (17). However, it requires moderate crew involvement, including microscopic interpretation, and does not reliably differentiate between viable and non-viable cells.

A second method is a direct viable count procedure (18). In this procedure, bacterial cells are incubated with yeast extract in the presence of naladixic acid to promote cell growth but prevent cell division. Following incubation, acridine orange is used to stain the cells. Only the enlarged cells are counted as "viable" or "substrate-responsive." "Non-viable" or "non-responsive" cells which remain normal in size can also be included for total counts. This procedure requires approximately 8-12 hours, moderate crew involvement, including microscopic interpretation, and may require advanced image analysis techniques for discrimination of responsive cells from mixed populations.

A third method is a modified membrane filtration procedure which incorporates a fluorogenic substrate in the medium. With bacterial growth and assimilation of the substrate, a rapidly detected fluorescent micro-colony is produced. This procedure requires approximately 6 hours and at the present time is only applicable to select physiological groups.

DEVELOPMENT OF A NEAR REAL-TIME MONITOR

The monitoring and evaluation of microbiological parameters onboard Freedom will be essential to the health of flight personnel and to the efficient and continual operation of the ECLSS system and subsystems. Even though the technology is available to accomplish the required tasks, the methodology needs to be further developed and modified to accommodate the conditions onboard the Freedom Station. Modifications to appropriate hardware are also required. Well-defined methods are employed on Earth to assess water and air quality with respect to microbiological standards. Some of these methods, such as membrane filtration could also be employed onboard Freedom with modification and miniaturization.

There is an inherent disadvantage in employing classical cultural methods for water and air analysis. All of these techniques require some incubation period during which time the organisms present can grow. This incubation period necessitates a lag time between the time of sampling and the time when results become available. This problem has been addressed somewhat on Earth with the development of rapid identification systems. These systems have been particularly useful in the identification of organisms from clinical samples where the rapid result is an obvious benefit. However, even using these rapid methods, a large number of cells are required.

With the employment of similar but modified methods onboard Freedom, there will be a period of uncertainty regarding the quality of water and air supplies with respect to microbiological content. If a microbiological problem should exist at the time of sampling, in all likelihood it will have intensified by the time the results are available. Additionally, unless rapid methods are employed, there is no assurance that the microbiological quality will be preserved until the results are available. At the time samples are collected, bacterial densities may be within specifications and a result obtained hours later may verify this. However, the microbiological content when the water is released for use may be considerably higher. Therefore, it is desirable to minimize this period of uncertainty.

In the past two decades, many advances have been made in the detection and identification of microorganisms. For the most part, these advances are oriented toward clinical rather than environmental aspects of microbiology. The sensitivities of these clinically applicable methods are approximately $1x10^3$ CFUs per mL. Although technology is not currently available for the rapid detection of microorganisms at the levels specified for Freedom (1CFU/100mL), many microchemical and microphysical techniques exist which, with proper modifications, might prove to be a rapid mechanism for the detection of low concentrations of microorganisms onboard the Freedom Station. With this in mind, we set out to define and evaluate these techniques.

TECHNICAL EVALUATION - Five concepts were originally identified as being applicable to microbiological monitoring in Space (Table 2). These concepts included cultural methods, single cell detection, biomolecular detection, specific product detection and general molecular composition. Within these concepts, twenty-eight specific methodologies were identified as having the potential for microbial detection and/or identification (Table 3). Each of these has appropriate applications for various types of monitoring. The major issues regarding assessment of viable microbial populations in any particular environment concern advantages, disadvantages, sensitivity, and applicability of the specific method in question.

We have attempted to address these issues in defining the criteria against which the potential methodologies can be evaluated. We have also identified criteria that specifically relate to use of these methods for monitoring in Space. Although criteria weighting and the rating of each method were somewhat subjective, great care was taken to maintain consistency throughout the evaluation process. Because laboratory comparison of all the methods was not practical, rating of the various technologies was based on data obtained from primary research publications, reviews and technical bulletins. The identification, definition and weighting of each of the criteria was accomplished by numerous lengthy discussions among the investigators.

Since no single method could meet all of the criteria established, a relative scoring analysis was used to discriminate among the evaluation criteria (Table 4). All of the methods were rated according to

TABLE 2 POSSIBLE METHODS TO EMPLOY FOR MICROBIOLOGICAL MONITORING IN SPACE

CONCEPT	RELATIVE SENSITIVITY	POTENTIAL METHODOLOGY	MAJOR ADVANTAGES	MAJOR DISADVANTAGES
MODIFICATION OF VIABLE PLATE COUNT	VERY HIGH	DETECTION OF MICROCOLONIES	HIGH SENSITIVITY SMALL SIZE OF INSTRUMENTATION	TIME LAG BEFORE RESULTS ARE AVAILABLE CREATION OF UNDESIRABLE MICROBIAL WASTE
SINGLE CELL DETECTION	POTENTIALLY VERY HIGH	FLUORESCENCE FTIR SPECTROPHOTOMETRY PARTICLE COUNTING	HIGH SENSITIVITY RELATIVELY SIMPLE SAMPLE PREP.	SPACE AND/OR POWER REQUIREMENTS FOR INSTRUMENTATION MAY BE RESTRICTIVE
BIOMOLECULE DETECTION	LOW TO HIGH DEPENDING ON SPECIFIC INSTRUMENTATION AND METHOD	VIS SPECTROPHOTOMETRY UV SPECTROPHOTOMETRY FLUORESCENCE SPECTROPHOTOMETRY RIA BIOLUMINESCENCE CHEMILUMINESCENCE	SIMPLE SAMPLE PREPARATION FOR SOME METHODS SPACE REQUIREMENTS MINIMAL FOR SOME METHODS	COMPLEX SAMPLE PREPARATION FOR SOME METHODS SPACE AND/OR POWER REQUIREMENTS MAY BE RESTRICTIVE FOR SOME METHODS NUMBER OF REAGENTS AND PROCESSING STEP MAY BE PROHIBITIVE FOR SOME METHODS GROWTH STEP MAY BE REQUIRED FOR SOME METHODS
SPECIFIC METABOLIC PRODUCT DETECTION	LOW TO HIGH DEPENDING ON SPECIFIC PARAMETER	GC GC/MS MS/MS	POTENTIAL OF HIGH SENSITIVITY POTENTIAL OF MINIMAL SAMPLE PREPARATION	SPACE AND/OR POWER REQUIREMENTS MAY BE RESTRICTIVE NUMBER OF REAGENTS AND PROCESSING STEP MAY BE PROHIBITIVE FOR SOME METHODS GROWTH STEP MAY BE REQUIRED FOR SOME METHODS
GENERAL MOLECULAR COMPOSITION	MEDIUM TO HIGH	GC GC/MS FTIR MS/MS	POTENTIAL OF HIGH SENSITIVITY MINIMAL SAMPLE PREPARATION HIGH POTENTIAL FOR IDENTIFICATION	SPACE AND/OR POWER REQUIREMENTS MAY BE RESTRICTIVE NUMBER OF REAGENTS AND PROCESSING STEP MAY BE PROHIBITIVE FOR SOME METHODS

TABLE 3 IDENTIFIED METHODOLOGIES

METHOD NUMBER	DESCRIPTION
1	VIABLE PLATE COUNT/MEMBRANE FILTER TECHNIQUE
2	VIABLE PLATE COUNT/SPREAD OR POUR-PLATE
3	DIRECT VIABLE COUNT (DVC)
4	LASER LIGHT SCATTERING
5	PRIMARY FLUORESCENCE
6	FLOW CYTOMETRY WITH FLUOROPHORE DETECTION
7	DIRECT EPIFLUORESCENT FILTER TECHNIQUE (DEFT)
8	IMMUNOFLUORESCENCE
9	ELECTRICAL IMPEDENCE/THE BACTOMETER
10	ELECTRONIC PARTICLE DETECTION
11	SCANNING ELECTRON MICROSCOPY
12	LIMULUS AMOEBOCYTE LYSATE ASSAY
13	BIOLUMINESCENCE
14	SPECIFIC ENZYME ASSAYS
15	FATTY ACID DETECTION
16	RAMAN SPECTROSCOPY
17	FTIR SPECTROSCOPY
18	GAS CHROMATOGRAPHY/MASS SPECTROSCOPY (GC/MS)
19	MASS SPECTROSCOPY/MASS SPECTROSCOPY (MS/MS)
20	VISIBLE SPECTROSCOPY
21	UV SPECTROSCOPY
22	PYROLYSIS
23	RADIOIMMUNOASSAY (RIA)
24	VOLATILE PRODUCT DETECTION/MEMBRANE FILTRATION
25	THERMAL PHOTOMETRY
26	CHEMILUMINESCENCE
27	MICROCALORIMETRY
28	POLYMERASE CHAIN REACTION

TABLE 4 CRITERIA USED FOR TECHNICAL EVALUATION

	WEIGHTING FACTOR
PRIMARY	
SENSITIVITY	5
TIME REQUIREMENTS (HOW RAPID)	5
TECHNOLOGICAL MATURITY	5
BROADNESS OF APPLICATION	5
SECONDARY	
SAMPLING	
PHYSICAL REMOVAL	
CREW INVOLVEMENT	3
NON-INVASIVE	5
	5
POST SAMPLING	
CONCENTRATION	1
MANIPULATION	1
ANALYSIS	
NONDESTRUCTIVE	
IDENTIFICATION	4
DISCRIMINATORY CAPABILITY	2
HARDWARE CONSIDERATIONS	4
INTEGRATION WITH OTHER METHODS	
INTEGRATION WITH CHEMICAL MONITOR	1
	1
POST ANALYSIS	
GENERATION OF WASTE MASS	
BIOHAZARD GENERATION	3
CHEMICAL HAZARD GENERATION	3
	5

the specified criteria relative to each other. The methods were first technically evaluated based on the primary criteria (Figure 1). Those methods receiving 75% of the total available points were technically evaluated using the secondary criteria. Methods receiving less than 75% of the total available points were eliminated. The results of the technical evaluation are presented in Table 5.

The five highest scoring methods from the technical evaluations were evaluated using established engineering and feasibility criteria (data not shown). These methods include laser light scattering, primary fluorescence, secondary fluorescence, electronic particle counting and volatile product detection (membrane filtration/mass spectroscopy). Each of these methods is briefly described below:

Laser Light Scattering - Laser light scattering is currently used for the detection and characterization of particulate contamination. This technique is applicable to both liquids and gases. Lasers can be selected to meet the different wavelengths which may be required for analysis. Simple modifications of this technology would allow spectrophotometric properties to be evaluated. The rapid response time of this technique allows for repetitive measurements which can improve precision and accuracy. Its major drawback is its limited capacity to discriminate between inanimate particles and bacterial cells. Using this method, real-time total-volume measurements are possible. In addition, this method may be applicable to cell sorting, which could be used to redirect the contaminated volume to another stream for reprocessing, sterilization or verification.

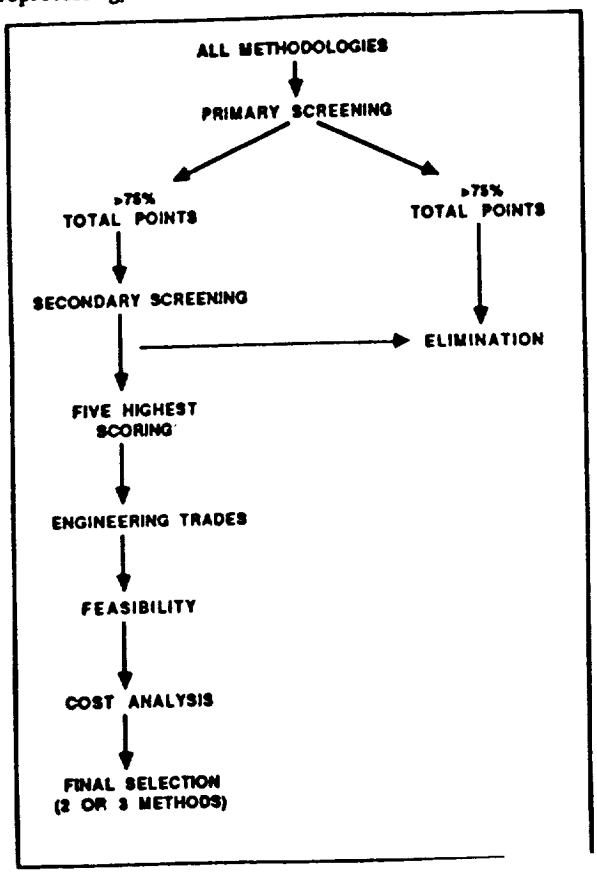

FIGURE 1 SCREENING OF CANDIDATE METHODOLOGIES

Primary Fluorescence - In some molecules the absorption of light radiation produces emission at a longer wavelength. A large number of molecules present in cells have fluorescent properties and could serve as "detection markers". Bacterial characterization is possible using known fluorescence decay times. Time-resolved fluorometry can detect these "markers" associated with living cells. Single-cell detection using primary fluorescence has been demonstrated, but is not routine (19,20). Real-time, total-volume analysis is feasible using this method. In addition, this method may also be applicable to cell sorting, which can be used to redirect the contaminated volume to the influent stream for reprocessing, sterilization or other verification.

Secondary Fluorescence - Secondary fluorescence involves the addition of a fluorophore to aid bacterial detection. A reagent mixture (cocktail) containing the fluorophore is added to the sample. A fluorimeter is used for detection. Reaction cocktails can be modified to analyze specifically for the physiological groups of interest, thus providing for a direct count of specific microorganisms. Differentiation of unbound and bound dye is based on the differences in decay times. Total volume analysis is not possible using this procedure, however. The secondary fluorescence procedure can be accomplished in near real-time and eliminates the need for crew involvement and microscopic evaluation, as required by the epifluorescence microscopic procedure. We estimate the time required between sample collection and analysis to be 30 minutes or less.

Volatile Product Detection - Volatile product detection would be accomplished using a hyphenated mass spectroscopic technique. In this method, a sample volume is concentrated using a 0.2 pore size membrane filter. The membrane filter is incubated under the appropriate conditions as required for the detection of a particular physiological group of interest. Over time, volatile products are analyzed from the head space. This analysis reveals the presence of microorganisms of a particular physiological group, with the time required for volatile product detection corresponding to the contamination level present. Identification of the volatile products provides information which characterizes the contaminant. This method is non-destructive, thereby allowing for verification of the results and subsequent processing of the contaminants. This method is directly applicable to liquids, gases and solids.

TABLE 5 SUMMARY OF THE TECHNICAL EVALUATION

METHOD NUMBER	DESCRIPTION	TECHNICAL PRIMARY*	TECHNICAL SECONDARY*	TOTAL POINTS
4	LASER LIGHT SCATTERING	70	122	192
10	ELECTRONIC PARTICLE COUNTING	70	110	180
6	PRIMARY FLUORESCENCE	60	114	174
8	SECONDARY FLUORESCENCE	60	82	142
24	VOLATILE PRODUCT/MS	60	76	136
13	BIOLUMINESCENCE	60	54	114
3	DIRECT VIABLE COUNT	60	53	113
1	MEMBRANE FILTRATION	46	67	113
26	CHEMILUMINESCENCE	56	60	116
7	EPIFLUORESCENCE	60	40	100
11	ELECTRON MICROSCOPY	60	36	106

* MAXIMUM AVAILABLE POINTS: PRIMARY 80 ; SECONDARY 144

Electronic Particle Detection - Electronic particle detection is by far the oldest of the candidate methodologies. This technique involves the detection and counting of particles as they temporarily alter the electric field through which they pass. Like laser light scattering, electronic particle detectors can determine the size and number of particles, but cannot distinguish between inanimate particles and living cells. An additional limitation is the small orifice size required for bacterial detection which results in limited flow and clogging of the orifice opening. This method is applicable to real-time total volume monitoring. Available instruments will need major modifications in design to be applicable for use in microgravity.

DISCUSSION

This study was undertaken in response to a strong concern that appropriate technologies should be developed for microbiological monitoring onboard Freedom. Accordingly, we defined five concepts applicable to microbiological monitoring. Twenty-eight specific methodologies within these concepts were evaluated for their potential use in Space. Of these, five were chosen as potential candidates for the development of a microbiological monitor for the Freedom station. The salient characteristics of these five candidate methodologies with regard to their application to microbial monitoring onboard Freedom are summarized in Table 6.

All of the techniques involve instrumentation or components which are available "off the shelf". This is extremely important, considering the long lead time required for the development of new equipment and it subsequent qualification as flight hardware. Four of the five methods would require little or no modification in equipment design. The one exception is electronic particle counting. Due to their complex mechanics and problems associated with calibration and maintenance available electronic particle counters are probably not compatible with microgravity conditions and other constraints of space vehicles without major modifications.

Four of the five candidate methodologies are real-time procedures. Analysis time was included in the primary screening criteria and minimization of this factor was weighted heavily in our evaluation scheme. Thus, only those techniques which could provide results in real-time or near real-time were considered as desirable methodologies.

Three of the five methodologies are routinely used for detecting particulate contaminants. The major problem with these techniques is that the instruments cannot inherently discriminate between inanimate particles and living cells. This problem, however, is not unique to specific techniques and there may soon be developments which will enhance the usefulness of these technologies for microbiological monitoring. This aspect is discussed in more detail later.

Four of the five candidate methodologies are non-destructive in nature. Organisms could therefore be archived and further analysis performed to identify or determine antibiotic sensitivity of the contaminating microorganism. We feel that this is a highly desirable characteristic.

Three of the five candidate methodologies are non-invasive. Non-invasive monitoring was heavily weighted in these evaluations for three reasons: 1) a non-invasive technique could potentially be used as a total volume monitor, thus providing an "absolute" number of contaminating organisms; 2) the possibility of contaminating the source is eliminated; and 3) the problems associated with sampling and sample size are eliminated.

With the possible exception of membrane filtration/volatile product detection and, to a limited extent primary fluorescence, none of the methods provide a great deal of information regarding the classification of the microorganism. It should be noted that the increased sensitivity which many of these methods demonstrate is off set by a significant reduction in the qualitative information obtained. It may be possible to increase the amount of qualitative information obtained by combining several methodologies, e.g., laser light scattering coupled with primary fluorescence. These two techniques could readily be incorporated into a microbial monitor in a way analogous to the optimization of techniques in fluorescence-activated cell sorters.

None of the methods listed above provide adequate detection of viral particles, although most will provide information regarding the other major groups of microorganisms, i.e., bacteria, fungi and protozoa. Of these groups, certainly bacteria and fungi will be the most predominant and probably of greatest concern onboard the Freedom Station.

We agree with previous authors (17) that microbial monitoring onboard Freedom should be viewed as a multi-tiered system. The first task which must be accomplished is to detect the presence of something

TABLE 6 COMPARISON OF FIVE CANDIDATE METHODOLOGIES

METHOD	MINIMAL MODIFICATION OF "OFF-SHELF" INSTRUMENTS	REAL-TIME DETECTION	NONDESTRUCTIVE	NONINVASIVE
LASER LIGHT SCATTERING	YES	YES	YES	YES
PRIMARY FLUORESCENCE	YES	YES	YES[a]	YES
SECONDARY FLUORESCENCE	YES	NO	NO	NO
VOLATILE PRODUCT DETECTION	YES	NO	YES	NO
ELECTRONIC PARTICLE DETECTION	NO	YES	YES	YES

[a] SUFFICIENT ENERGIES OF SHORT WAVELENGTHS WOULD BE DESTRUCTIVE

undesirable; in this case, a particle in the size range of a microbial cell. Once this is accomplished, more discreet analysis would yield additional information regarding the contaminant. Ultimately, positive identification of contaminants may be required in-flight on a contingency basis. Other, archived samples could be returned to Earth for further analysis. The information obtained from these samples would be invaluable in the design of future spacecraft or space-residence ECLSS systems and in the definition of microbial monitoring systems and protocols in those structures.

SUMMARY

Public health concerns on Earth have required that certain microbiological parameters be monitored, including the safety of potable water supplies. These same concerns demand a high priority on Space Station Freedom as well, since the inhabited modules will provide limited space. Treatment operations under these conditions must process higher input loads in order to maintain optimal conditions. In addition, since the Freedom Station will operate as a marginally closed environmental system, any source of potential microbial hazard must be identified as soon as possible in order to minimize risk to the crew as well as to structural or operational systems. Thus, real-time, continuous microbial monitoring would be ideal. Many different technologies can be identified which are applicable to microbiological monitoring. However, only a handful of these can meet the stringent sensitivity and time constraints necessary. In fact, no single method currently available can meet all criteria established for a real-time microbiological monitor. Trade-offs in sensitivity, rapid detection, automation, sampling, etc., are usually made at the expense of other criteria, such as discriminatory capability, identification potential and non-destructive analysis techniques.

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APPENDIX E

SUBCONTRACTOR PROGRESS REPORTS

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Subject: Real-Time Microbiological Monitor (RTMM) - First Progress Report
November 7, 1988

This document is the first of a series of progress reports on the preliminary development of a Real-Time Microbiological Monitor (RTMM) for space vehicle application under University of Alabama-Huntsville contract No. 5-31886. Spectroscopic technology related to this application is reviewed, and specific recommendations with regard to further investigations and proof of concept evaluations are made.

RTMM Requirements

The following set of RTMM requirements have been established by UAH/NASA:

1. It must be adaptable to water, air and surfaces.
2. It must be reliable and rapid.
3. It should be self-contained and require minimal crew involvement. Ideally, it should be an automated expert system providing for a signal indicating unacceptable conditions.
4. It should be both sensitive and selective. False positive results are undesirable.
5. It should lend itself to improvements and modifications toward both a quantitative and qualitative monitor which can evolve to a completely automated expert system.
6. It should provide real time or near real time data.

Sensitivity of the instrument must be sufficient to detect:

1. 1 colony forming unit (cfu) of total bacteria per 100 ml in water
2. 1,000 cfu/m^3 in air
3. 100 cfu/25cm^2 on surfaces

Methodologies

Of the potential methodologies described in the UAH proposal (page 8) to NASA dated March 17, 1988, two are believed to be compatible with real time detection and identification:

1. Biomolecule Detection
2. General Molecular Composition

These two characteristics both permit immediate measurement without the need for extensive sample handling, filtering, reagent reactions or other time-intensive procedures. As the names suggest, biomolecule detection is most useful in detection only application, and molecular composition in identification applications. Detection methods will be discussed first.

Biomolecule Detection

Five spectroscopic technologies were considered for initial RTMM evaluation:

1. Ultraviolet/Visible Absorbance
2. Near Infrared/Infrared Absorbance
3. Fluorescence
4. Phosphorescence
5. Resonance Raman

Three of the above technologies were eliminated from further consideration for the following reasons:

1. Ultraviolet/Visible Absorbance
   not sufficiently sensitive at very low levels of micro-organism concentration
2. Near Infrared/Infrared Absorbance
   also not sufficiently sensitive
3. Phosphorescence
   requires either very low temperatures, water removal or oxygen removal

The two remaining technologies seem particularly well-suited to both detection and identification applications. Fluorescence seems the more practical approach in the near future, but Resonance Raman has distinct advantages and should become more practical as the state of the art of elecrro-optical technology develops.

Fluorescence for Detection and Identification

In some molecules, absorption of light radiation produces light emission at a longer wavelength. This phenomenon is a form of luminescence known as fluorescence or phosphorescence. Fluorescence differs from phosphorescence mainly in its persistence with fluorescence having a much shorter decay after a pulse of light. Fluorescence decay times are measured in nanoseconds (1-100 nanoseconds) while phosphorescence lifetimes last as long as 100 seconds. Since phosphorescence requires special environmental preparations, it does not lend itself to real-time on-line measurement.

A large number of molecules present in cells have fluorescent properties and could serve as "detection markers". Some of these molecules and their excitation and emission wavelengths are tabulated below:

Biomolecule	Excitation Peak (nm)	Emission Peak (nm)
Tyrosine	275	303
Tryptophan	287	348
Serotonin	295	330
Phenylalanine	260	282
NADH-NADPH	340	460
ATP,ADP	272	380

Of the above molecules, NADH/NADPH is recommended as the bacteria marker of choice for the following reasons:

1. All living cells contain NADH/NADPH.
2. The peak excitation wavelength in the 340nm region is consistent with the use of a nitrogen laser as an excitation light source.
3. The pulsed nature of the nitrogen laser with its high peak powers (4 kilowatts) provides a potential for high sensitivity.
4. At the same time, the low average power (millowatts) minimizes energy usage on a space vehicle and allows for a compact air-cooled design.
5. Nitrogen lasers are currently available at reasonable cost in compact form and are highly reliable in operation.
6. The short pulse width of the nitrogen laser allows for the use of time-resolved fluorescence analysis to identify as well as detect all known bacteria.
7. The use of NADH/NADPH as a bacteria detector and cell concentration marker has already been proven in industrial applications.[1]
8. The use of a nitrogen-laser based time-resolved fluorometer for bacteria detection and identification has been demonstrated in the laboratory.[2,3]
9. OptoMed Technology personnel have extensive experience with nitrogen laser based time-resolved fluorometers and associated pattern recognition techniques needed for bacterial identification.

Bacteria detection using time-resolved fluorometry will be based on the time-integration of a number of laser pulses so as to achieve extremely high levels of sensitivity. A boxcar average or gated integrator will be used in the proposed instrumentation.

Bacterial identification will be based on the measurement of a series of fluorescence lifetimes over a range of emission wavelengths as shown in Table I.[3] Each type of bacteria will be characterized by a feature vector of decay times stored in memory of the fluorometric instrument. A measurement vector will then be compared with the feature vector library using traditional pattern recognition techniques (e.g. minimum distance classifier, nearest neighbor) to identify the type of bacteria.

A description of a proposed Time-Resolved Fluorometer (TRF) for RTMM proof of concept demonstration is provided in Appendix I of this report.

Resonance Raman for Bacterial Identification

The proposed Real-Time Time Resolved Fluorometer (RTTRF) described in the previous paragraph is currently recommended as the best current approach to RTMM consistent with the present state of the art in spectroscopic instrumentation. It is important to understand, however, that published research has revealed the existence of Resonance Raman Spectroscopy as a technique potentially superior in the identification of bacteria.

Raman spectroscopy is a technique based on the measurement of light scatter in which a small portion of this scattered light is shifted in wavelength based on the chemical structure of the molecules involved. If a monochromatic laser light source is used, each molecule will emit a characteristic Raman-shift spectrum at wavelengths longer than the exciting wavelengths. Raman spectra are often characterized by sharp peaks at particular wavelengths that permit easy identification of individual molecules. Nelson and his associates at the University of Rhode Island have shown in a series of experimental studies[4] that Resonance Raman techniques may be used to identify bacteria.

Resonance Raman Spectroscopy is a form of Raman Spectroscopy in which the exciting wavelength is at a resonance point so that the output light emission is increased by 3-4 orders of magnitude over basic Raman emission producing signal levels comparable to fluorescence spectroscopy. Non-resonance Raman signals are too small for practical applications in RTMM.

Resonance Raman methods for bacterial identification are most effective using ultraviolet excitation wavelengths under 260nm. Recent research has emphasized sub-260nm Raman excitation with outstanding results. Resonance Raman is not currently recommended for RTMM application for the following reasons:

1. Light Source
   -The only presently suitable light source for Resonance Raman is a costly and complex dye laser that is not suitable for RTMM.
2. Lack of Detection Experience
   -There is recorded experience of the use of Resonance Raman for bacteria detection. The use of this somewhat complex technique for simple detection would be very questionable.

The future of Resonance Raman for RTMM will depend on:

1. The availability of simple reliable light sources
2. An increased emphasis on bacteria identification

Two approaches to simpler light sources are on the horizon:

1. Frequency-doubled Argon Laser
   New crystals will permit high efficiency frequency-doubled operation of an Argon Laser at 244nm.
2. Laser Simulator
   OptoMed is developing a laser simulator for operation at 253nm.

It is not possible to predict the time availability of either of these light sources at this time. Future progress reports will update the status of Resonance Raman as development proceeds.

Conclusions and Recommendations

Preliminary investigation indicates that a nitrogen-laser based time-resolved fluorometer for RTMM should be pursued for proof of concept demonstration. RTTRF has potential for both bacteria detection and identification. A description of RTTRF is included as Appendix I of this report.

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November 7, 1988

APPENDIX I

A TIME-RESOLVED FIBER FLUOROMETER (TRFF) FOR THE REAL-TIME MICROBIOLOGICAL MONITOR (RTMM)

The proposed Time-Resolved Fiber Fluorometer in its various configurations, will have application to microbiological measurements in water, air and on surfaces. All configurations use the same basic time-resolved fluorometer and all use remote fiber-linked measurements. The configurations differ primarily in the form of the optrode used to collect light for instrumental analysis. An optrode (or probe) is a device that transmits excitation light to a media and receives fluorescence light from the media. All instrument configurations are also based on the principles of Remote Fiber Fluorescence Spectroscopy (RFFS) which will now be described to clarify the functions of the proposed TRFF.

RFFS is a technique for remote spectral measurement of fluorescent conmpounds using a fiber optic link. A basic RFFS System, as shown in Figure 1, consists of a light source (laser or lamp), a chopper, illumination optics, a fiber cable, collection optics and a photodetector. RFFS is particularly well-suited to microbiological detection and identification because most micro-organisms have fluorescence properties. Unfortunately, the fluorescence spectra of these microorganisms overlap significantly so that sophisticated analytical techniques are required to develop a composition profile of a multi-bacteria mixture. Such analytical techniques encompass two primary areas:

1. Multicomponent Spectral Analysis
2. Time-Resolved Decompostion Analysis

Multicomponent spectral analysis is required to resolve overlapping biochemical spectra into their individual spectral components. A typical set of excitation and emission spectra for a biochemical compound is shown in Figure 2. These spectra are for a single compound. In a typical microbiobiological environment there are a multitude of such compounds and a complex spectral pattern that can only be resolved using various forms of multicomponent analysis. Such analytical techniques extend from multiple correlation analysis to identify optimal wavelengths for each compound to stepwise regression analysis for composition function formulation. More advanced techniques include partial least squares regression, factor analysis and other variants under the general category of multicomponent analysis.

Appendix I

Even the most advanced multicomponent analysis techniques, however, may fail to determine the composition of the more complex formulations. To determine these, it is necessary to introduce a third dimension, time. Fluorescent compounds are also characterized by varying time decay (lifetime) functions. Decay times vary from less than one (1) nanosecond to over one hundred (100) nanoseconds. A typical fluorescence time decay function is shown in Figure 3. If the target compound is excited by a short pulse of light, it will then decay at a rate characteristic of the compound.

To provide for fluorescence time decay measurements, the basic fiber fluorometer of Figure 2 must be expanded into the Time-Resolved Fiber Fluorometer of Figure 4. This expanded system includes a nitrogen laser as the light source, fiber fluorometer optics, a decay time computer to measure the decay time function and a microcomputer to perform decomposition analysis.

In a microbiological environment with its multiple chemical compounds, the decay time function is complex. The nature of the function is illustrated by a three-component mixture in Figure 5. Decomposition analysis provides for the time-resolution of a complex time decay function into its component parts. Biotronics has developed a program called LYNREG, a piecewise linear regression technique, to perform such analyses.

In the general situation for on-line microbiological analysis, spectral analysis and time-decay analysis merge into multi-component, time-resolved fluorometry. Time-resolved fluorometry is especially appropriate when excessive overlapping interferences exist between the fluorescent excitation and emission spectra of a multiple-compound mixture. The third variable of decay time often allows for the separation of interfering compounds and general background fluorescence from the compound of interest.

Time-resolved fluorometry is a two-stage process consisting of lifetime measurements and decomposition analysis.

1. Fluorometric Lifetime Measurements
   A series of fluorometric lifetime measurements are made of samples at different emission wavelengths to form a data matrix (D). This matrix can be decomposed into two factors: (A), which contains the spectra of the individual components in its columns, and (C),which contains the time behavior of the components in its rows.
   $(D) = (A)(C)$
   (D) is a wxt matrix where the w is the number of wavelengths observed in spectrum and t is the number of time intervals at which spectra are obtained.
   (A) is a wxn matrix containing the n individual components in its columns.
   (C) is a nxt matrix containing the time behavior of the n components in its rows.
2. Decomposition Analysis
   The resolution of the mixture requires that the data matrix (D) be decomposed into (A) and (C) in order to identify the individual components by their spectra and decay kinetics.

OptoMed has had experience in both the instrumentation and the analytical aspects of time-resolved fluorometry, so that it is able to assist a user in the practical aspects of both laboratory experimentation and on-line monitoring.

With the basic theory of operation established, fiber fluorometry as it applies to microbiological monitoring will be examined at three levels of complexity. In the first level, Basic Fiber Fluorometry (BFF), a single fluorescent variable is measured to determine the presence of bacteria without regard to the detailed composition of the microbiological mix. At the next level of complexity, Multiple-Component Fiber Fluorometry (MCFF), fluorescence is measured at multiple wavelengths to determine the composition of the process and the identification of individual bacteria. Finally at the highest level of complexity, Time-Resolved Fiber Fluorometry (TRFF), measurements in the time domain as well as the frequency domain permit determination of more complex microbiological mixtures. Each of these three systems will now be examined with an emphasis on hardware and software features of each system.

Basic Fiber Fluorometer Operation

A basic fiber fluorometer (BFF) is illustrated in Figure 6. This diagram illustrates the Biotronics BI-401 Remote Fluorometric Analyzer. The BI-401 implements the BFF originally shown in Figure 1. A xenon arc lamp provides the light source. Single bandpass interference filters are used for excitation and emission. The filtered light is coupled to a fiber cable and transmitted to the optrode (probe) in the process tank. Fluorescent light is transmitted back to the instrument where it is detected by a photomultiplier tube and converted to digital form for signal processing.

Signal processing computations are very simple in the BFF. Calibration of the instrument is accomplished using a linear function of the form:

$$C_s = m(F_s - F_1) + C_1$$

where

C_s - Concentration of sample

m - Slope parameter

F_s - Fluorescence, sample

F_1 - Fluorescence, low standard

C_1 - Concentration, low standard

Calibration determines the parameters m (slope) and C_1 (concentration, low standard) of the linear function where:

$$m = dc/(F_h - F_1)$$

m - Slope parameter

$dc = C_h - C_1$ - difference in high and low standard concentrations

F_h - Fluorescence, high standard

F_1 - Fluorescence, low standard

The concentration of the fluorescent compound (or bacterial cells) is then determined from the linear function. The BFF functions quite well in a process with a single fluorescent compound. Microbiological processes, however, are typified by multiple fluorescent compounds. The BFF has important application, however, in the RTMM. In many instances, multiple compound fluorescent emissions will provide a changing fluorescence intensity at a single wavelength that will "track" the prescence of bacteria without regard to type. In other words, the presence of bacteria and the concentration of cells will be indicated by the fluorescence intensity at the selected wavelength. While this approach is not able to identify bacteria, it is useful in basic detection of micro-organisms.

Multiple-Component Fiber Fluorometer (MCFF) Operation

The MCFF, referencing Figure 6, hardware differs little from the BFF. The BI-401 functions as an MCFF with the addition of the filter wheel shown in 1C5 in the diagram. The filter wheel allows for fluorescence measurements at multiple excitation and emission wavelengths. This information along with the implementation of multiple component analysis software allows for limited identification of bacteria as long as the number and degree of spectral overlap between fluorescent compounds are not too complex. Processes with many bacteria with extensive spectral overlap require the next level of system complexity: time-resolved fiber fluorometry.

Time-Resolved Fiber Fluorometer (TRFF) Operation

TRFF requires a significant increase in both hardware and software complexity. The special hardware and software features of a TRFF are described below:

1. Light Source
   The extremely short decay times of fluorescent bacteria require a pulsed light source with a nanosecond-level pulse width. The nitrogen laser fulfills this requirement. The ultraviolet excitation wavelength of the nitrogen laser (337.1 nm) also corresponds with the excitation waveband of most bacteria.

2. Decay Time Computer
   A special high speed computer is required to determine the time decay function of the fluorescent mix. It includes a set of programmable comparators and a time interval counter. It is illustrated in Figure 8. The decay time function is determined with a resolution of 0.1 nanoseconds.

3. Decomposition Software
   Multiple wavelength time decay functions serve as inputs to a software package that determines the composition vector of the process mix.

The TRFF represents the highest level of complexity, but it is also the ultimate in performance of the various fiber fluorometer systems. It should be used only identification of bacteria is a system requirement. BFF and MCFF are preferable for simple bacteria detection.

OptoMed Technology and Fiber Fluorometry

OptoMed has had extensive experience with all three complexity levels of fiber fluorometry. This experience has been augumented by parallel experience with fiber-based infrared absorption spectrometry, which uses similar hardware and software techniques, particularly in the area of multicomponent analysis.

In fiber fluorometry and related technology, OptoMed has been involved with four system developments:

1. BI-401 Fiber Fluorometric Analyzer
   The BI-401 is an on-line fluorometric analyzer capable of operating at both the BFF and the MCFF levels. Designed for an industrial environment, the BI-401 is housed in a NEMA 4 enclosure and features armored cable to protect the external fiber link. A vital element in any fiber analyzer is the optrode or probe that interfaces with the process in either a tank or pipe configuration. Both types of optrodes are illustrated in Figure 9. Equally important as the optrode itself is its application in the process network and its role in calibration. Such an application is illustrated in Figure 10.

2. BI-103 Reflective Fluorometer/Phosphorimeter
   This instrument is for laboratory application, but it involves a fiber optic link and a different (reflective) optrode. It also uses a pulsed xenon light source which allows for application in lifetime measurements in phosphorimetry. Phosphorimetric lifetimes are longer than fluorometric lifetimes and do not require a special decay time computer. It uses a 4x40 liquid crystal display to provide operator prompting messages and numeric output. Excitation and emission filters are manually changeable and allow for multiple wavelength testing in the laboratory.

3. Decay Time Fluorometer (DTF)
   Designed for laboratory use, the DTF provides for single excitation/emission wavelength operation. The DTF incorporates the elements illustrated in Figure 4. The optical layout is shown in Figure 7 with the special elliptical curvette holder to provide for more efficient light collection.
   The two primary proprietary components of the DTF include the Decay Time Computer shown in Figure 8 and the LYNREG software package for the piecewise linear regression of the decay time function into its constituent components.

4. BI-400 Infrared Fiber Analyzer
   The BI-400, like the BI-401, is an on-line fiber analyzer. It is based on infrared absorption measurements rather than fluorescence. It is mentioned here only because it is an on-line fiber analyzer similar to the BI-401, and it does use multicomponent analysis to separate overlapping spectra.

Given the wide range of instrument development and production experience in a variety of fiber fluorometric instruments, it is now pertinent to define the remaining development necessary to develop a comprehensive set of instruments capable of operating at all three levels of fluorescence measurement: BBF, MCFF and TRFF.

In summary, Biotronics has developed each of the major hardware components of a total system including:

1. On-Line Fiber-Optic Link and Fluorescence Optrode (BI-401)
2. Laser-Based, Time-Resolved Fluorometer (Decay Time Fluorometer)
3. Decay Time Decomposition Software (Decay Time Fluorometer)
4. Multicomponent Analysis Software (BI-400)

The proposed TRFF development project will involve the integration of the above four elements into a new TRFF System. This system may be used to demonstrate the feasibility of TRFF for RTMM in the Space Station.

Price and Delivery

Prices

Time-Resolved Fiber Fluorometer	-	$49,375
Data Logger and Deconvolution/Pattern Recognition Software	-	2,300
		$51,675

Delivery

180 days after receipt of order

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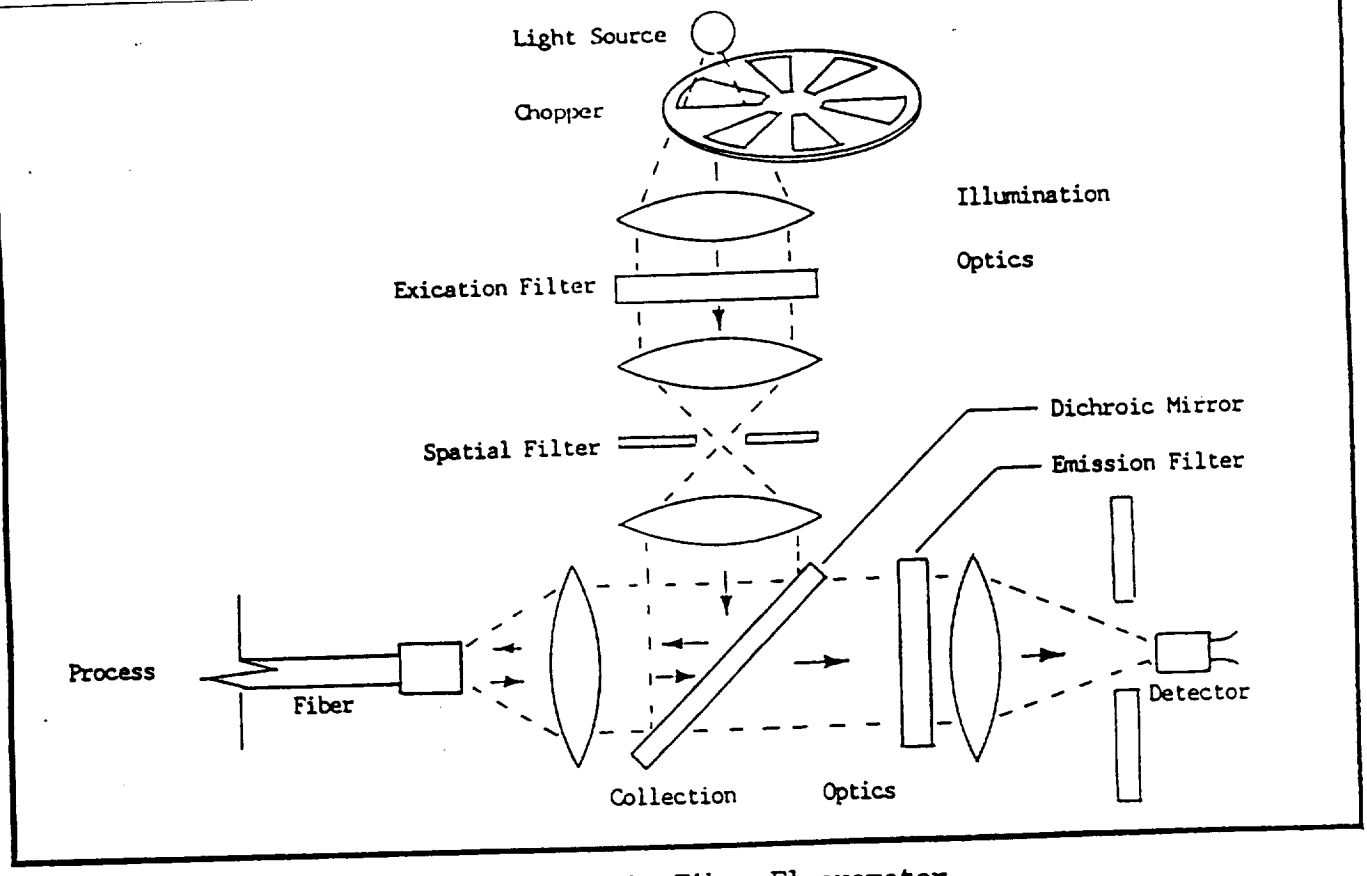


Fig. 1 Basic Fiber Fluorometer

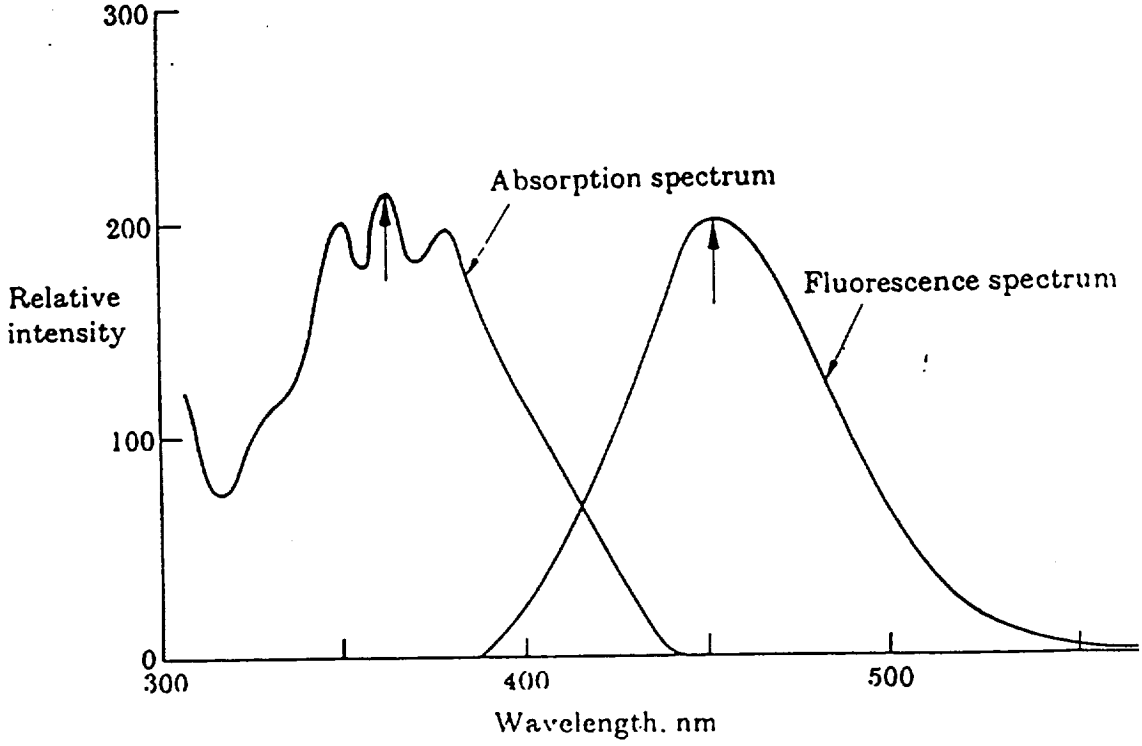


Fig. 2 Absorption and Fluorescence Spectra

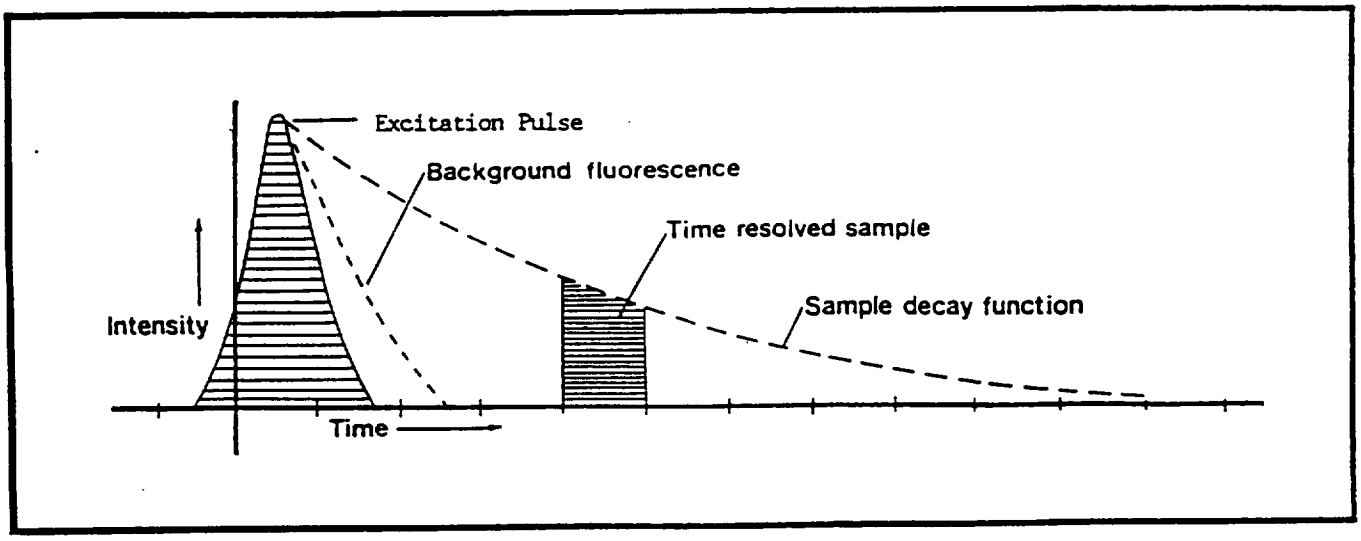


Fig. 3 Fluorescence Time Decay Function

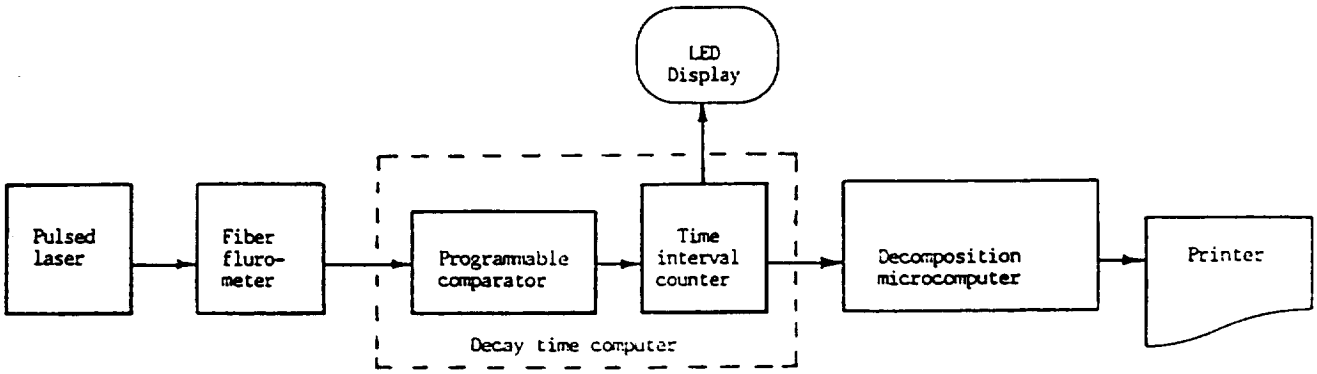


Fig. 4 Time Resolved Fiber Fluorometer System

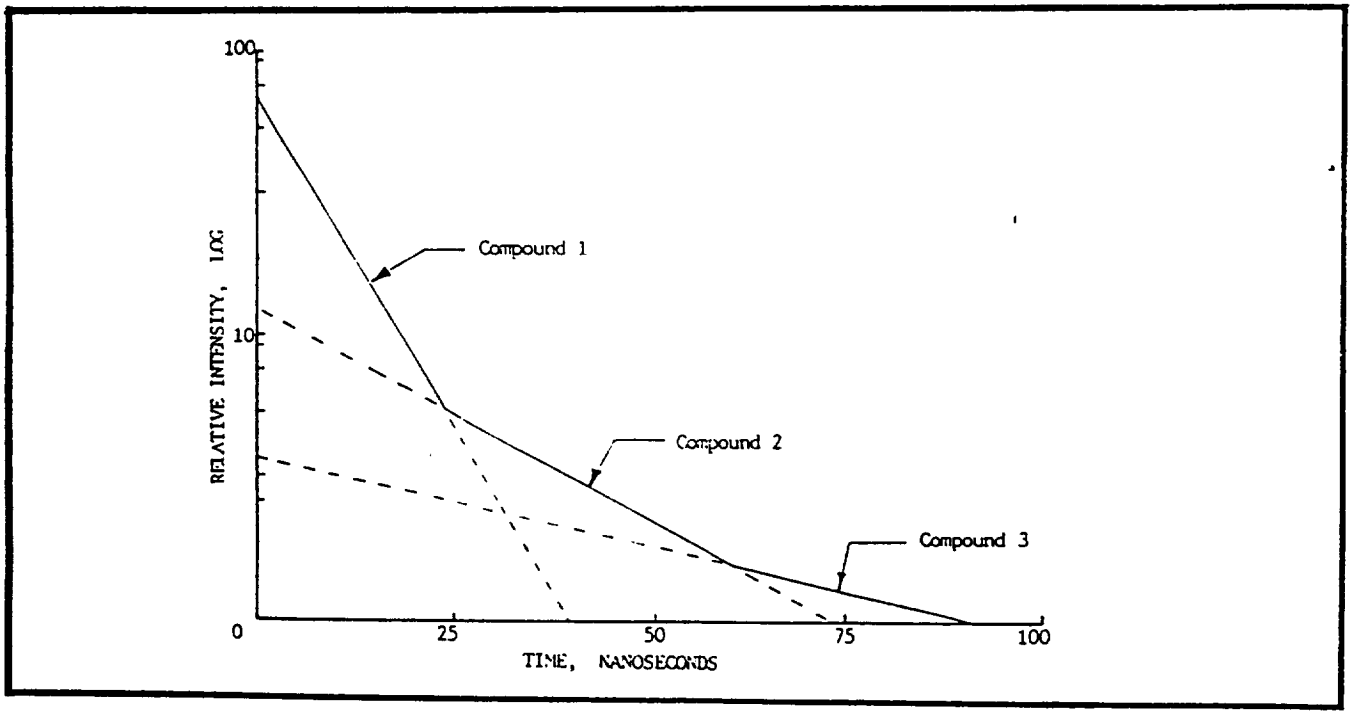
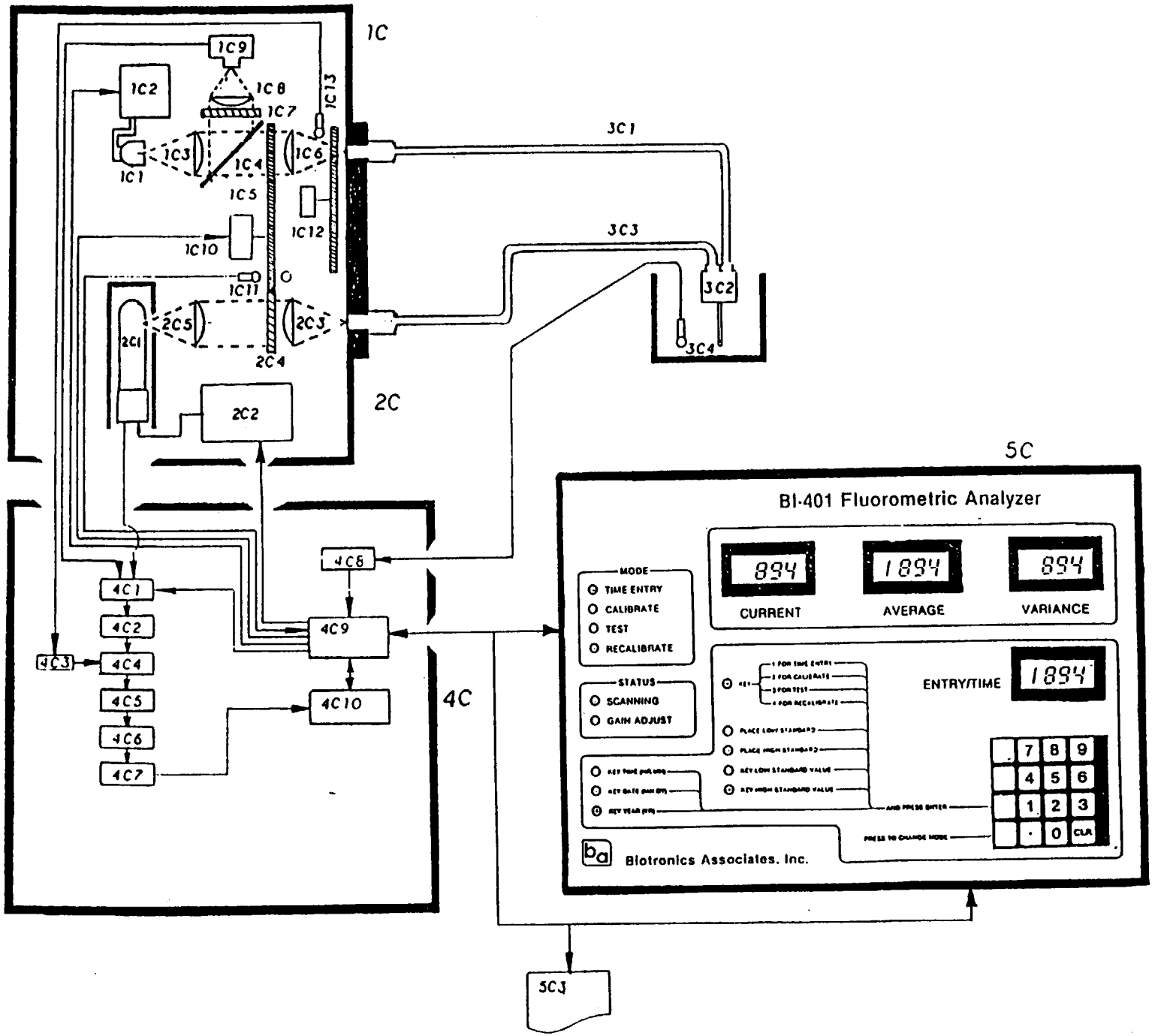


Fig. 5 Three Component Mixture Time Decay Function



1C SOURCE OPTICS ASSY
1C1 ARC LAMP
1C2 LAMP POWER SUPPLY
1C3 COLLIMATING LENS SYSTEM
1C4 BEAM SPLITTER
1C5 FILTER WHEEL
1C6 FIBER LAUNCH LENS
1C7 BROAD BAND UV FILTER
1C8 DETECTOR LENS
1C9 REFERENCE PHOTODIODE
1C10 FILTER WHEEL MOTOR
1C11 FILTER WHEEL POSITION SENSOR
1C12 CHOPPER
1C13 CHOPPER FREQUENCY SENSOR

2C RECEIVER OPTICS ASSY
2C1 PMT TUBE IN HOUSING
2C2 PMT POWER SUPPLY
2C3 FIBER COLLIMATING LENS
2C4 FILTER WHEEL
2C5 PMT LENS

3C PROBE ASSY
3C1 SOURCE FIBER
3C2 PROBE TIP
3C3 TARGET FIBER
3C4 TEMPERATURE PROBE

4C MICROCOMPUTER AND CONTROLLER
4C1 REFERENCE/TARGET MULTIPLEXER
4C2 BANDPASS FILTER
4C3 WHEEL POSITION CONDITIONER
4C4 LOCK IN AMPLIFIER
4C5 LOW PASS FILTER
4C6 SAMPLE AND HOLD
4C7 A/D
4C8 A/D
4C9 I/O INTERFACE
4C10 MICROCOMPUTER

5C OPERATOR PANEL

5C3 MATRIX PRINTER

Fig. 6 Basic Fiber Fluorometer (BI-401 Block Diagram)

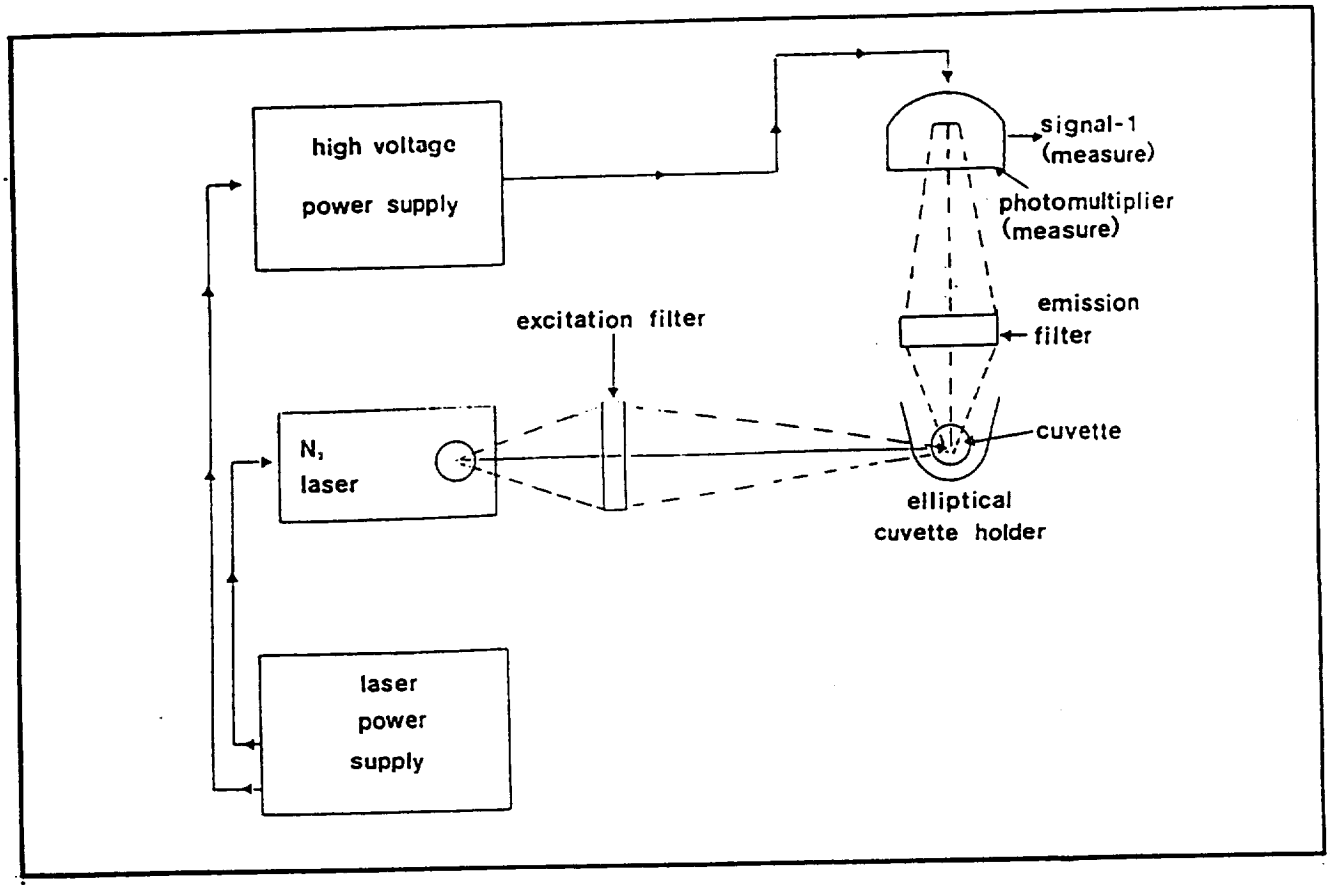


Fig. 7 Optical Diagram, Time-Resolved Fluorometer

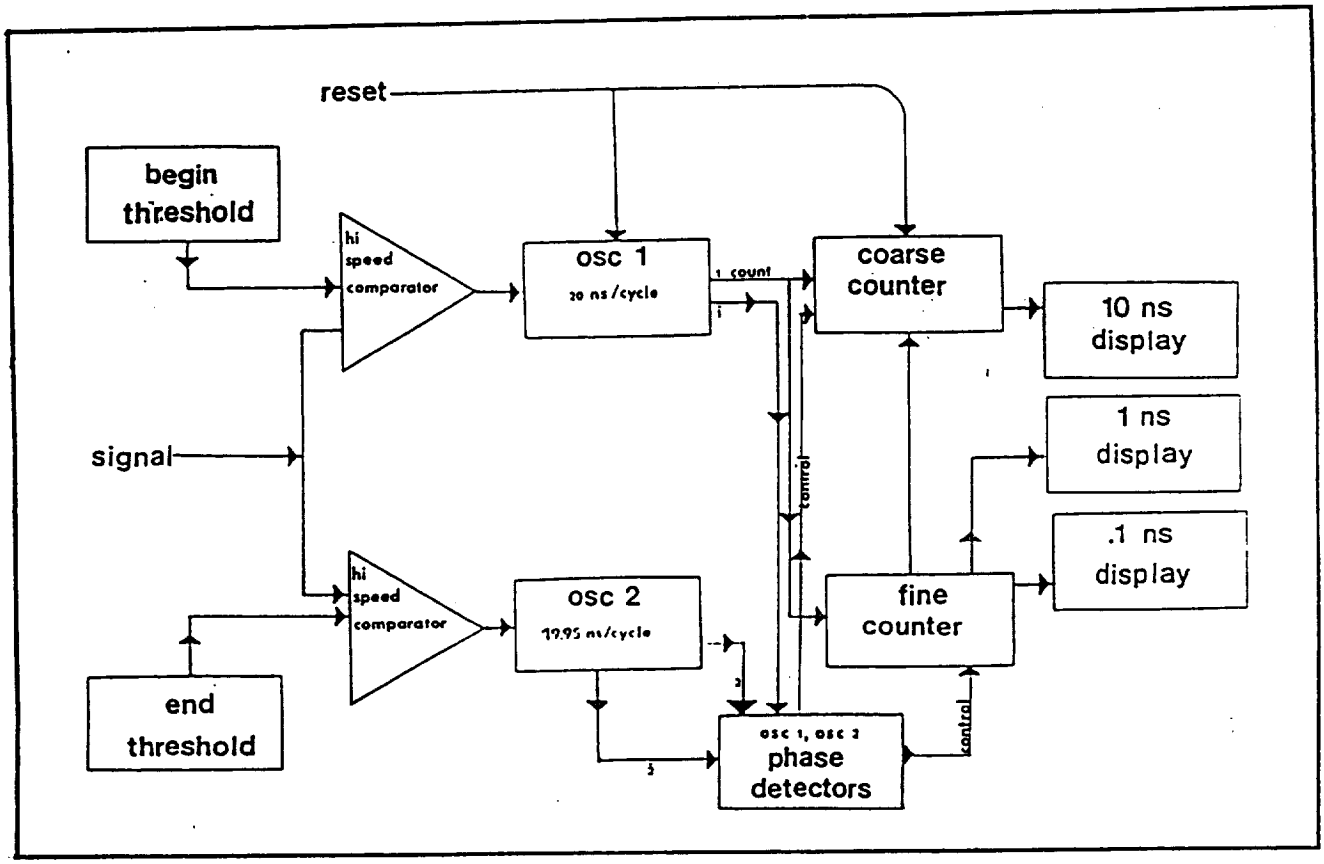
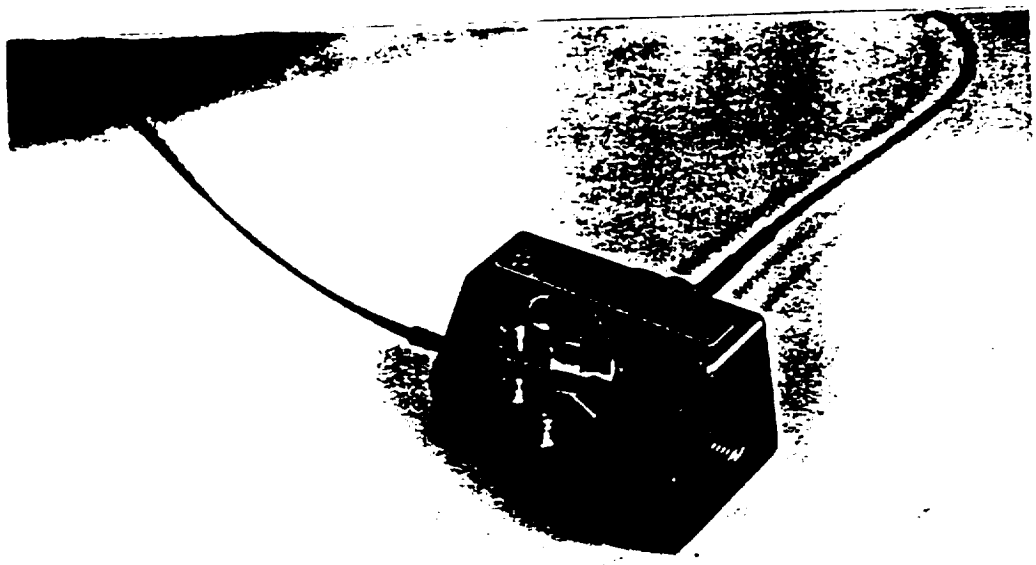
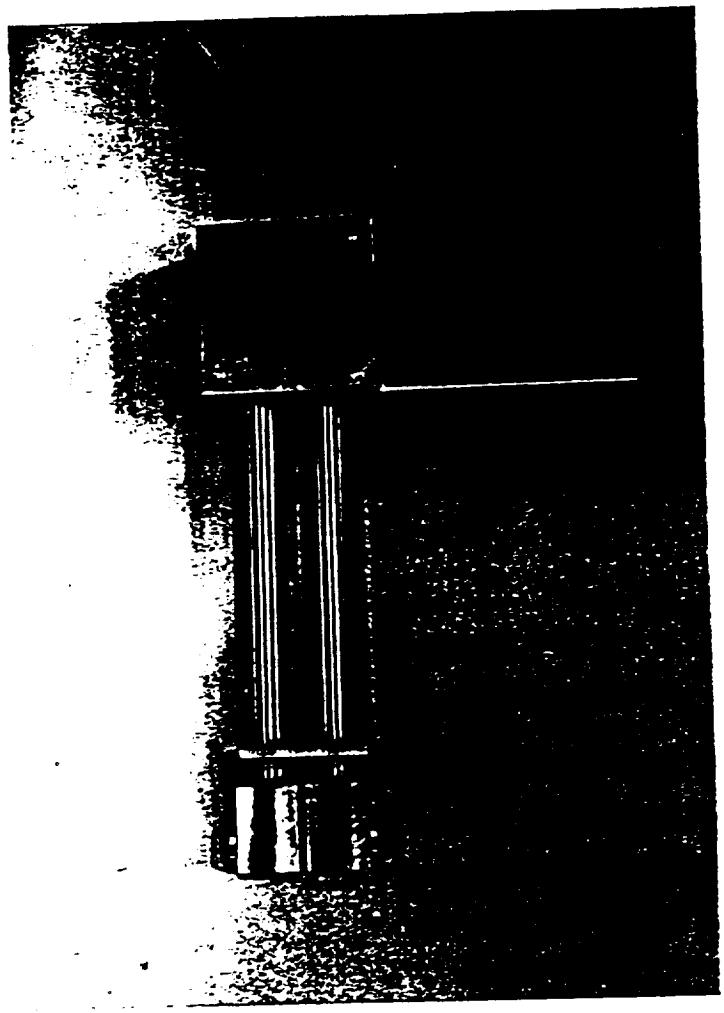


Fig. 8 Block Diagram, Decay Time Computer



Pipe Optrode



Tank Optrode

Fig. 9 Fluorescent Optrodes

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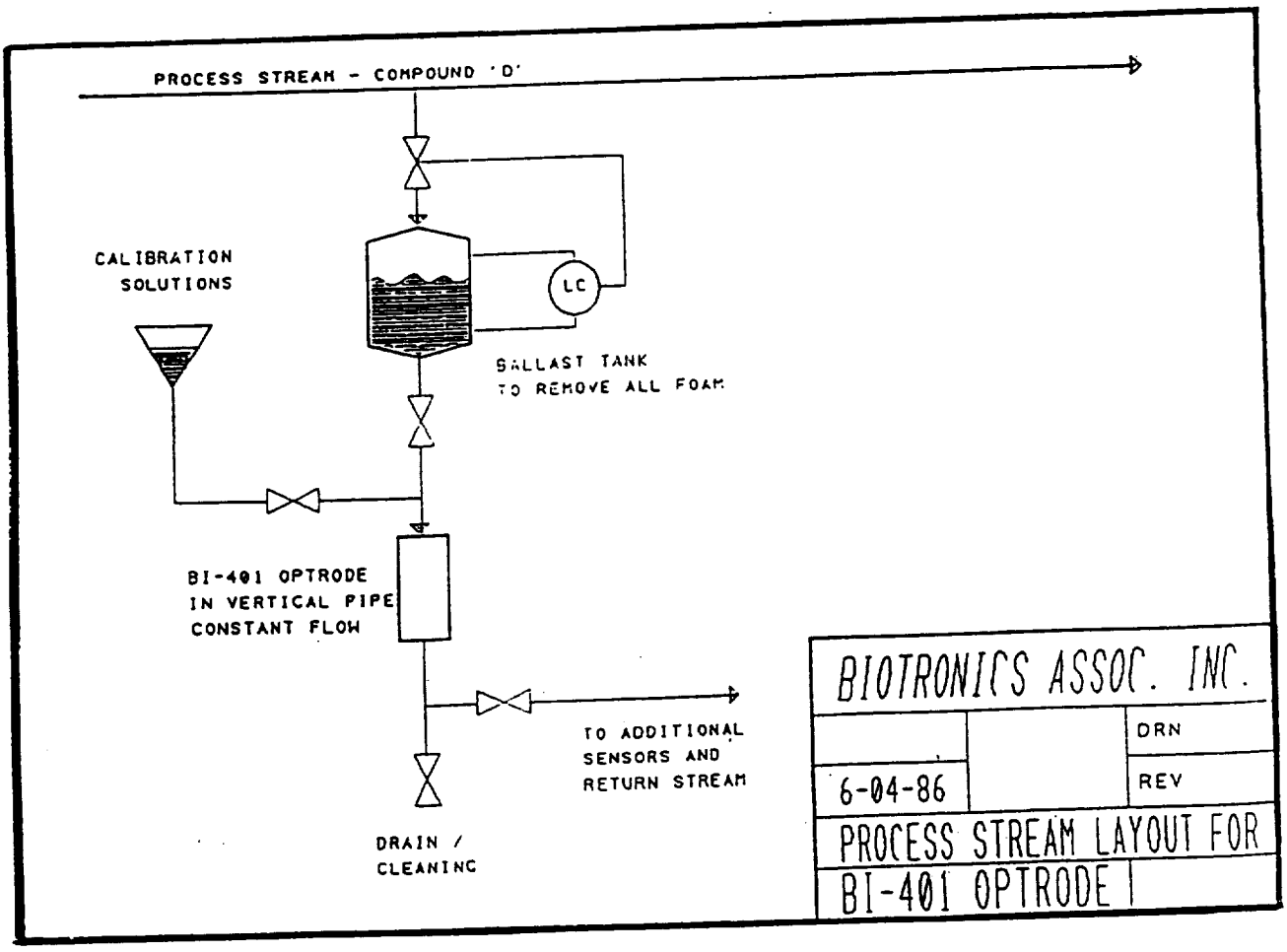

Fig. 10 Process Stream Layout for BI-401 Optrode

December 27, 1988

Subject: Real-Time Microbiological Monitor (RTMM)
Second Progress Report - December 27, 1988

This document is the second of a series of progress reports on the preliminary development of a Real-Time Microbiological Monitor (RTMM) for space vehicle application under University of Alabama-Huntsville contract No. 2-21013. This report investigates the primary design parameters and components of the Time-Resolved Fiber Fluorometer (TRFF), the instrument recommended for RTMM in the first progress report dated November 7, 1988. The writer and personnel associated with OptoMed Technology have had extensive experience with both basic and time-resolved fluorometry. This report will reflect this experience in its discussion of the design issues and potential operating characteristics of a TRFF for RTMM.

TRFF Design Review

Summarizing previous discussions in the first progress report, the six major components of the TRFF are:

1. Laser-Based Fluorometer Optics
2. On-Line Fiber Optic Link and Fluorescence Optrode
3. Decay Time Computer
4. Microcomputer and Associated Interfaces
5. Decomposition Software
6. Multicomponent Analysis and Pattern Recognition Software

The first three of these major components will be discussed in this report in terms of their effect on overall TRFF performance and the present state-of-the-art of current technology. The last three will be discussed in the next progress report.

Laser-Based Fluorometer Optics

The optical layout of a basic fiber fluorometer is shown in Figure 1. All of the elements shown in Figure 1 are applicable to a TRFF except for the chopper. Pulsed light fluorometers do not require a chopper wheel or its associated lock-in amplifier. These elements are appropriate only in a continuous measurement environment. The emission filter will be expanded to a filter wheel capable of providing eight separate wavebands since the identification method requires fluorescence measurements at multiple wavelengths. The emission filter wheel will also be augumented by a second fixed high-pass filter in series. This proprietary filter is of the liquid chemical type and blocks all potentially stray light coming from the laser light source from detection by the photomultiplier tube detector. This "blocking filter" essentially prevents any light from wavelengths less than 350nm from entering the detection optics. Such stray light elimination is extremely crucial to optimal TRFF sensitivity performance.

The other three critical optical components are the laser light source, the light collection optics and the photomultiplier detector. The nitrogen laser has evolved technically from a research tool requiring an external gas supply and a pump into a self-contained operational laser light source requiring neither.

A nitrogen laser, the VSL-337, available fromn Laser Science, Inc. of Cambridge, Massachusetts, is totally self-contained and measures only 9 3/4" X 4 1/2" X 2 1/2". It pulses with an output peak power of 40 kilowatts and 3 nanoseconds duration. This high peak power in conjunction with the liquid blocking filter discussed previously provides for a very high sensitivity system. Pulses may be generated at rates up to 20 pulses per second. The unique feature of the VSL-337 is its disposable sealed nitrogen plasma chamber integrated in a single compact module with the energy depostion switch and energy storage capacitors. This chamber contains all the active components of the laser with inherent finite lifetimes in a single disposable cartridge. The cartridge can be replaced in minutes virtually eliminating down-time when a back-up module is available. Since module lifetime is more than 30 million pulses, replacement will be infrequent. Few lasers seem better suited to the space environment.

Detection efficiency is also enhanced by the use of a high efficiency ellipsoidal mirror in the collection optics. This mirror, as shown in a previous Biotronics' application in Figure 2, serves to increase light collection efficiency, and studies have shown the ellipse to be the optimal mirror shape[1] for this purpose.

Photomultiplier tubes represent a mature technology but one that still is the method of choice for sensitive fluorescence detection. A previous TRFF design used the RCA Model 1P28 PMT, but an extensive review of current PMT product offerings will be conducted for the new TRFF development. OptoMed will also use its PMT circuit experience in developing an optimal PMT configuration.

Fiber Optic Link and Fluorescence Optrode

A fiber-linked optrode is necessary for the TRFF to provide the flexibilty needed for combined water, air and surface measurements. Water measurements may be accomplished using a pipe optrode as shown in Figure 3. In this configuration excitation light is beamed into the process stream. Fluorescence light is then detected by collection optics oriented at a 135^{o} angle to the excitation light beam. This configuration has previously been shown to be very suitable for low absorbance liquids such as water. To obtain the optimum efficiency for single cell light collection, it will probably be necessary to integrate an ellipitical mirror collector into the optrode itself. It may even be necessary to mount the PMT itself in the optrode to avoid fiber attenuation losses. Fiber bundles and large diameter (1mm) fibers will be explored as alternatives.

Air measurements could also be accomplished using a pipe optrode approach in a forced air return (or returns) in the space station. Considerable experience with fluorescence and Raman shift measurement of gases is recorded in the literature.[2] A number of applications use a nitrogen laser as the light source.[3] One of these applications involved the detection of bacteria in the atmosphere. A separate version of the TRFF could scan the air in a given open space if required.

Surface measurements can be made with a reflective optrode as used in the Biotronics' BI-103 Reflective Fluorometer as shown in Figure 4. The same basic instrument would be used with a modular detachable fiber probe. This reflective measurement utilizes a fiber bundle for the fiber cable connection.

Decay Time Computer

The purpose of the Decay Time Computer is to generate a series of time interval measurements that may then be averaged to produce a composite decay time function (Figure 5). This function is then processed by the microcomputer in a combined deconvolution/multi-component analysis to produce a spectral lifetime feature vector for bacterial identification (Figure 6). A typical lifetime feature vector set is shown in Table I. This data was developed by Nelson and his associates at the University of Rhode Island.[4]

The software development details of decomposition, multicomponent and pattern recognition analysis will be discussed in the next progress report. Here we will confine our interest to the operation of the Decay Time Computer (DTC).

It should be emphasized initially that the DTC operates as a peripheral under the control of the microcomputer. With reference to Figure 7, a block diagram of the DTC, the input threshold values are provided by the microcomputer. The outputs of each time interval measurement are also sent to the microcomputer for further signal processing.

The internal operation of the DTC is easily explained. Given a Begin Threshold reference signal, the PMT output signal will start oscillator 1 which generates a pulse every 20 nanoseconds. This threshold is established near the top of the decay curve. These pulses accumulate in the coarse counter which has a resolution of 5 nanoseconds. A series of End Threshold reference signals are programmed to activate oscillator 2. When the laser pulse amplitude decreases to a given value below the Begin Threshold (start) level, oscillator 2 is started. This oscillator generates pulses at a faster rate (19.95ns/cycle) and eventually "catches up" with oscillator 1. Catch-up time is a function of the fractional time difference inside the 5 nanosecond coarse counter interval. If the end threshold signal occurs coincident with an oscillator 1 pulse, catch-up time is zero. If the end threshold is halfway through the coarse cycle, then 2.5 nanoseconds is added to the coarse indication to obtain the total time interval. In total, the two counters measure the time interval to the nearest 0.1 nanosecond.

Decay time function measurement accuracy is improved by extensive averaging. Two averaging strategies are possible:

1. Point averaging
2. Function averaging

In point averaging, multiple readings are averaged at each end threshold level. In function averaging, multiple sweeps of the time interval range are averaged. Better performance seems to favor function averaging since it is less affected by short term instrument drift.

Previous experience with the DTC indicates that the primary source of inaccuracy arose from variation in the amplitude of the nitrogen laser pulses. Some improvement will result from inmproved stability in nitrogen lasers. Function averaging, as opposed to point averaging, also will minimize laser amplitude variation effects. To achieve the ultimate in decay function measurement accuracy, however, OptoMed has developed a new method of pulse normalization that essentially removes pulse variation as an important source of error in time-resolved fluorometric analysis. Incorporation of this technique in the new design should provide the required data for accurate identification of microorganisms.

Conclusions and Recommendations

Preliminary investigation of the hardware components required for a new TRFF for RTMM application indicates that such an instrument could be developed and delivered in a 4-5 month time period with a high probability of successful operation. Previous experience of OptoMed personnel in this technology makes such a quick response possible.

The investigation of TRFF will continue in the next time period with detailed consideration of software and analytical questions.

Kenneth J. Schlager, Ph.D., P.E.

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February 14, 1989

Subject: Real-Time Microbiological Monitor (RTMM)
Third Progress Report - February 14, 1989

This report is the third of a series of progress reports on the preliminary development of a Real-Time Microbiological Monitor (RTMM) for space vehicle application under University of Alabama - Huntsville contract No. 17102-89. This document relates to the software requirements of the Time-Resolved Fiber Fluorometer (TRFF) previously recommended in the two earlier progress reports dated November 7, 1988 and December 27, 1988. The report also outlines the technology requirements for a back-up aided fluorescence measurement method based on the use of fluorochromes that may be required to achieve the ultimate sensitivity in the detection of microorganisms.

TRFF Design Review

The second progress report emphasized consideration of the hardware needs of the TRFF including:

1. Laser-Based Fluorometer Optics
2. On-Line Fiber Optic Link and Fluorescence Optrode
3. Decay Time Computer

This report will outline requirements for the second half of the six major components of the TRFF:

1. The Microcomputer and Associated Coprocessors and Interfaces
2. Decomposition Software
   -to operate on the decay time function and determine its separate components
3. Pattern Recognition Software
   -to identify the microorganisms

Since there is no assurance that the TRFF will achieve the ultimate sensitivity in water measurements of 1 colony forming unit (cfu) per 100 milliliters, extensive thought has been given to a back-up procedure involving the use of fluorochromes (dyes) that in an off-line manual procedure would allow for even more sensitive detection.

Microcomputer and Associated Coprocessors and Interfaces

Since the computations associated with decomposition of the fluorescence decay time function are quite intensive, some attention should be given to the choice of a microcomputer. Although the nature of the computational workload will depend somewhat on the deconvolution algorithm selected, all methods will require a degree of numerical processing power.

In the interest of compatability with existing software, a PC-compatible microcomputer is preferable. A PC-compatible not only makes best use of existing software, but it also allows for easy expansion of computing power in the form co-processors and special digital signal processing circuits to perform fast Fourier Transforms and other special functions. Future real time situations could result in a short computation cycle that could require some form of parallel processing. "Transputer" (Inmos trade name) boards can now be added to a 8088/80286/80386 microcomputer that provides a form of high speed parallel processing.

Microcomputer interfaces will be needed on the TRFF for the following functions:

1. Operator Control and Display
2. Printer (optional)
3. Analog output (4-20MA) for control signal (optional)
4. Discrete output(s) for alarms (optional)
5. Decay time computer (DTC) interface

All of the interfaces except the last one are conventional and are easily implemented with off-the-shelf components. The human interface requirements of NASA, however, should be factored into the choice of an appropriate control/display panel. The DTC interface will be an active one since data acquisition depends on close cooperation between the DTC and the microcomputer.

Decomposition Software

The decay time function is a time-amplitude function resulting from a combination of the laser excitation and the fluorescence emission processes. Determination of the individual component time-amplitude functions needed for pattern recognition requires a deconvolution of this multi-component function.

A wide variety of mathematical methods have been used to perform this deconvolution ranging from graphical resolution of two-component mixtures[1] to large matrix manipulations of combined wavelength-decay time data.[2] Other methods have been based on the method of moments[3,6] and various forms of regression analysis.[4]
Associates of the writer have developed a program based on piecewise linear regression analysis called LYNREG.[5] Some methods utilizing Fourier Transform techniques are not well documented in the literature, but they may offer the best promise for real time computation using high speed digital signal processing. At the present time, plans call for a dual approach using previously proven LYNREG with attempts at improvements in processing speed using a coprocessor and a second approach featuring the Fast Fourier Transform.

Whatever the approach finally used, the output of the program will be a decay time (lifetime) versus wavelength matrix similar to that shown in Table I except that typically only one or two bacterial elements will be present in the matrix. This matrix serves as the input for the pattern recognition program.

Pattern Recognition Software

Three factors can make the recognition of specific microorganisms challenging in TRFF applications:

1. Pattern Distance Separation
   The geometric or Mahalanobis distances between the fluorescence vectors of different bacteria may be small.
2. Measurement Noise
   Noise levels in fluorescence measurements in combination with small vector separation may result in low signal to noise ratios.
3. Library Size
   Time will be required to accumulate a library of bacterial fluorescence templates for storage in the TRFF. In early use of the system, all bacteria will be detected, but some will not be identified for lack of a template in the library.

In spite of possible reduced signal-to-noise ratios, OptoMed is well prepared to apply the best in current pattern recognition technology to the identification of microorganisms based on their fluorescence lifetime vectors. Both classical Bayesian methods as well as the newer Neural Network techniques will be evaluated.

Classical pattern recognition techniques are well documented in standard texts.[7,8] A preferred classical method is Fisher's Linear Discriminant which is the optimal rule for pattern classification for the normal, equal covariance case.[9] In experimental data collection, it is both possible and convenient to test the assumptions of normality (normal distribution of patterns) and equal covariances. Experience here in near infrared spectroscopic analysis indicated that both assumptions are quite valid in many analytical chemistry applications. Only experience will tell how valid they are in microbiologic identification.

Should the assumptions of the Fisher linear discriminant prove not tenable in TRFF measurements, exploration of new pattern recognition techniques in neural networks (also called neurocomputing) will be required. The writer presently is quite involved in research in this area, and the same networks currently under development could easily be transferred to TRFF application. There has recently been an explosion of interest in neural networks, and the literature is extensive. A new society, The International Neural Network Society, was formed in 1988. It is not appropriate to provide a tutorial on neural networks in this report. A copy of a well composed small tutorial booklet on neural computing has been included with this report. This booklet entitled Teaching Your Computer to Learn is a good introduction to neural networks. A series of more advanced references in neural computing is also included with this report.[10, 11, 12, 13]

The power of neural networks lies in their potential capability to classify overlapping noisy patterns that are resistant to classical methods. If TRFF statistical assumptions prove invalid and lifetime patterns are noisy and overlap significantly, neural networks may be the only feasible approach to recognition. In any event, OptoMed is well prepared to implement neural network methods in TRFF should they be required.

Fluorochrome-Aided Detection

It is indeed fortunate the same Time-Resolved Fiber Fluorometer (TRFF) used to detect and identify microorganisms in the native fluorescence mode has equal powers of discrimination in an aided fluorescence mode of operation. Aided fluorescence makes use of organic compounds of high quantum efficiency known as fluorochromes to aid in the detection of microorganisms undetectible in the native fluorescence mode. Such low level detection is based on the change in fluorescence lifetime that occurs when the fluorochrome is bound to a substrate such as a microorganism. In the bound state the quantum efficiency of the fluorochrome changes resulting in a longer lifetime for the bound fluorochrome. For example, pyrene sulfonic acid exhibits a 62 nanosecond lifetime in water which increases to 140 nanosecond in a bound state.[14] Similarly pyrene buteric acid exhibits a 140 nanosecond lifetime in water and a 200 nanosecond lifetime in a bound state.[15]

Although there are a wide variety of known fluorochromes[16], OptoMed[17] recommends the application of pyrene derivatives such as pyrene buteric acid for the following reasons:

1. Long Lifetime
   Pyrene derivatives have comparatively long lifetimes in the 50-200 nanosecond range and these lifetimes change significantly in the bound state.
2. Excitation Wavelength
   The maximum excitation of pyrene derivatives occur in the 335-340nm region which is ideal for the 337nm nitrogen laser.
3. Clinical Experience
   The writer and his associates at OptoMed have had successful experience with pyrene derivatives as fluorochrome tags in fluoroimmunoassay. Pyrene derivatives are the odds-on favorite of most clinical researchers in time-resolved fluorometry.

The only other known organic competitive fluorochrome suitable for nitrogen laser excitation is dansylchloride (DNS-Cl). This compound has a maximum excitation wavelength in the 340nm region, but its average lifetime is only 14 nanoseconds and is much less sensitive to substrate bonding.

Rare earth metal chelates have been used as probes in time-resolved fluorometry and have much longer (50-1000 microseconds) lifetimes than pyrene derivatives. Rare earth metals such as europium, however, are expensive and often unstable in compound form. For this reason, they seem less desirable than pyrene derivatives. Europium chelates do excite well at 337nm, however, and could be applied to the TRFF without hardware or software changes.

Whatever fluorochrome probe is used, the principle of detection is the same in the TRFF and is illustrated in Figure 1. A laser pulse of width W excites the target sample. The unbound fluorescence of the fluorochrome and the native fluorescence of the bacteria decay so that by time T_3 they can no longer be detected. The bound fluorochrome, however, will still have a significant intensity at time T_3 and may be detected until time T_4. This integrated T_3-T_4 signal provides the basis for aided microorganism detection.

The outstanding feature of this whole fluorochrome process is that it can be performed with the same TRFF instrument with only software changes. The Decomposition/Pattern Recognition software would be replaced with a time-gated integration (boxcar integrator) synchronized with the delayed T_3-T_4 time interval.

In summary then, the TRFF will be operable in three separate modes:

1. Native Fluorescence Detection Mode
   -for NADH-NADPH measurement at Excitation - 337nm and Emission - 460nm
2. Native Fluorescence Identification Mode
   -for bacterial identification measurements
   Excitation - 337nm
   Emission - 400nm to 500nm at 20nm intervals
3. Aided-fluorescence Detection
   -for low level detection based on fluorochrome (pyrene derivative) lifetime changes
   Excitation - 337nm
   Emission - 392nm

In the future it might also be possible to develop a fourth mode based on the differences of lifetime changes for different bacteria.

Conclusions and Recommendations

The software for the TRFF instrument is now existent from previous developments at OptoMed. It remains only to integrate it into the new TRFF instrument. The back-up method of Fluorochrome-aided detection of microorganisms is feasible again based upon previous experience with probes using pyrene derivatives.

This preliminary investigation of the TRFF will conclude in the next period with a comprehensive detailing of the work tasks and the hardware/software/biochemical components needed for a prototype TRFF instrument.

Kenneth J. Schlager

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REAL-TIME MICROBIOLOGICAL MONITOR (RTMM)

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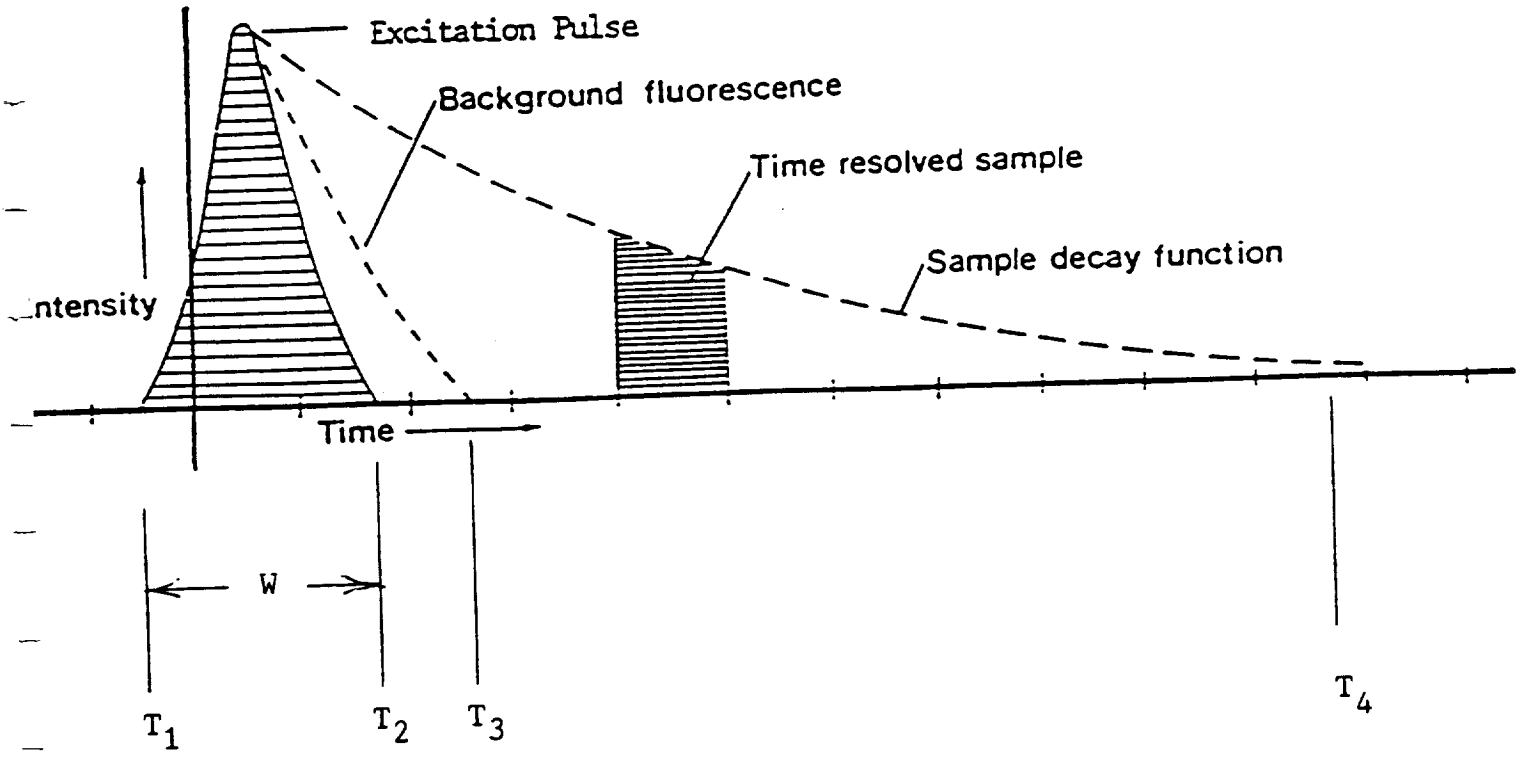

Fig. 1 Fluorescence Time Decay Function

TABLE I. Average fluorescence lifetimes of four bacteria obtained (400-500 nm).

Bacteria	λ nm	Average lifetimes (ns) 400	420	440	460	480	500
Enterobacter cloacae		(8.4)	(8.0)	(7.4)	(6.8)	(5.4)	(5.2)
Escherichia coli		(7.5)	(6.7)	(6.0)	(6.0)	(4.8)	(4.5)
Pseudomonas fluorescens		(8.5)	(7.9)	(9.3)	(9.3)	(8.1)	(8.0)
Staphylococcus epidermidis		(7.6)	(6.5)	(5.9)	(5.6)	(5.0)	(4.0)

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February 23, 1989

To: Mr. Melvin V. Kilgore, Jr.
Consortium for the Space Life Sciences
The University of Alabama in Huntsville
Huntsville, Alabama

Subject: Real-Time Microbiological Monitor (RTMM)
Addendum to Third Progress Report on Proof of Sensitivity Instrumentation (POSI)

This report is a supplement to the Third Progress Report (February 14, 1989) relating to a proposal for a Proof of Sensitivity Instrumentation (POSI) to demonstrate the probable sensitivity of the future Time-Resolved Fiber Fluorometer (TRFF). POSI is intended to demonstrate sensitivity performance in two of the three modes of TRFF operation:

1. Mode 1 - Native Fluorescence Detection
2. Mode 3 - Aided-Fluorescence Detection

The Native Fluorescence Identification Mode (Mode 2) will not be demonstrated in POSI because of its complex instrumentation requirements. The intent of the POSI program is to achieve the lowest level of microorganism detection in each of the two modes with the ultimate detection goal of 1 colony forming unit (cfu) per 100 milliliters of water.

POSI Description

The POSI system will consist of the following major components shown in Figure 1:

1. Nitrogen Laser
   to generate 3 nanosecond, 4 kilowatt peak power pulses at a rate of up to 20 pulses per second at a wavelength of 337.1nm
2. Fiber Optic Link
   to transmit the laser light pulses to the sample compartment simulating the fiber connection in the TRFF
3. The Sample Compartment
   to allow for illumination and fluorescence emission of the liquid sample in the cuvette cell
4. Detector/Preamplifier
   A photomultiplier tube (PMT) detector and preamplifier for fluorescent light detection and amplification
5. Boxcar/Gated Integrator
   to average or provide gated integration of detected fluorescent light pulses
6. Analog/Digital Converter and Digital Display
   to display the value of the fluorescencence measurement

POSI Operation

POSI operation makes use of the previously described equipment to operate in the following modes:

1. Native Fluorescence Detection Mode (Mode 1)
   A water sample containing microorganisms is placed in the cuvette in the sample compartment. The sample is radiated with laser light at 337.1nm and generates fluorescence with a peak at 460nm. For high sensitivity, POSI is operated using the gated integrator option. Samples of varying cell counts will be tested to determine the lowest level of detection.
2. Aided-Fluorescence Detection Mode (Mode 3)
   Operation is similar to Mode 1 except that a pyrene derivative reagent is placed in the sample cuvette and a waiting period is required for cell binding. Fluorescence measurement is then accomplished using a fixed T_1-T_3 delay time prior to gated integration to allow for the T_1-T_3 decay of native and unbound fluorescence light. Gated integration is then performed in the T_3-T_4 time interval. Varying sample concentrations are detected to determine ultimate sensitivity.

Cost and Schedule

Costs involved in implementing the POSI fabrication and test sequence include:

1. POSI Design Costs
   -to design the electro-optical and electronic components of POSI and prepare an overall system schematic
2. POSI Fabrication Costs
   -to purchase parts and assemble the POSI system
3. POSI Testing Costs
   -to test the set of samples in both modes

Total costs of all of the above activities are estimated at $17,195. A detailed cost breakdown is included in Appendix I.

The schedule for POSI design, fabrication and testing is estimated at 10 weeks.

Kenneth J. Schlager

APPENDIX I

POSI COST ESTIMATE

Materials and Parts		
1.	VSL-337 Nitrogen Laser	$3,300
2.	Low RFI Housing	795
3.	PMT Housing and Power Supply	500
4.	Boxcar/Gated Integrator	2,000
5.	Other Optical Components	1,500
6.	Analog/Digital Converter and Display	1,200
7.	Other	1,200
		$10,495
Labor		
1.	Electro-Optical Engineer 40 hours @$30/hour	1,200
2.	Systems Engineer 40 hours @$40/hour	1,600
3.	Biochemist 30 hours @$30/hour	900
		$3,700
Contingency		$3,000
	Total	$17,195

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March 25, 1989

To: Mr. Melvin V. Kilgore, Jr.
Consortium for the Space Life Sciences
The University of Alabama in Huntsville
Huntsville, Alabama

Subject: Real-Time Microbiological Monitor (RTMM)
Addendum to Third Progress Report on Proof of Concept
Instrumentation (POCI)

The request for on-line versus off-line proof of concept instrumentation requires a modification to the original addendum. Cost increases result from the engineering design effort and more complicated testing required.

POCI Description

3. Substitute Fiber Probe for Sample Compartment to allow for illumination and fluorescence emission of the flowing liquid in the fiber-linked optrode.

Cost and Schedule

Change the total costs to $24,795 (see Appendix I/Revised)

Kenneth J. Schlager

APPENDIX I

POCI COST ESTIMATE

Materials and Parts		
1.	VSL-337 Nitrogen Laser	$ 3,300
2.	Low RFI Housing	795
3.	PMT Housing and Power Supply	500
4.	Boxcar/Gated Integrator	2,000
5.	Other Optical Components	1,500
6.	Analog/Digital Converter and Display	1,200
7.	Other	3,500
		$12,795
Labor		
1.	Electro-Optical Engineer 100 hours @ $30/hour	3,000
2.	Systems Engineer 80 hours @ $40/hour	3,200
3.	Biochemist 60 hours @ $30/hour	1,800
		$8,000
Contingency		$4,000
	Total	$24,795

Note: The title was changed to Proof of Concept Instrumentation (POCI) at your request.

March 27, 1989

To: Mr. Melvin V. Kilgore, Jr.
Consortium for the Space Life Sciences
The University of Alabama in Huntsville
Huntsville, Alabama

Subject: Real-Time Microbiological Monitor (RTMM)
Fourth Progress Report-March 27, 1989

This document is the fourth and final of a series of progress reports on the preliminary development of a Real-Time Microbiological Monitor (RTMM) under University of Alabama-Huntsville contract no. 17102-89. This document summarizes plans for the implementation of a complete laboratory prototype RTMM based on the technology discussed in the three previous reports.

Since the publication of the third progress report on February 14th, an addendum to that report has been published relating to the proposed Proof of Concept Instrumentation (POCI) to demonstrate the sensitivity of the Time-Resolved Fiber Fluorometer (TRFF) in the two detection (not identification) modes. A revised version of that addendum is included with this report that expands the scope to include on-line detection of water-based microorganisms. After a clarification of the differences between POCI and the Laboratory Prototype, this report will detail plans for the development of the prototype.

POCI and the Laboratory Prototype TRFF

The POCI is intended to demonstrate the sensitivity potential for the detection of microorganisms using the TRFF approach. It is not concerned with the identification of microorganisms, nor is it a prototype instrument. Only two of the six major components of the TRFF will be present in the POCI:

1. Laser-Based Fluorometer Electro-Optics
2. On-Line Fiber Optic Link and Fluorescence Optrode

The above components will be augmented by laboratory test instruments to provide time delays, gated integration and output values.

Missing in POCI will be the other four major components of the TRFF:

1. Decay Time Computer
2. Microcomputer and Associated Interfaces
3. Decomposition (deconvolution) Software
4. Multicomponent Analysis and Pattern Recognition Software

The above four components are necessary only for the identification mode of TRFF operation.

Laboratory Prototype Development

Although there are a multitude of tasks involved in developing and testing a Laboratory Prototype TRFF, this report will discuss only the primary tasks that are crucial to the success of the project. A comprehensive list of all project tasks is included in Appendix I attached.

Three primary tasks will determine the success of the Laboratory Prototype TRFF:

1. Decay Time Computer Development
2. Deconvolution Software Development
3. Pattern Recognition Software Development

Although OptoMed personnel have had extensive experience in all three of the above tasks in previous instrumentation developments, improvements will be required to achieve all of the objectives of the RTMM program. Critical issues for each of these developments are discussed in the paragraphs below.

Decay Time Computer Development

OptoMed personnel successfully developed a decay time computer in the 1979-1982 time period as part of a Time-Resolved Fluorometer used for fluoroimmunoassay. This DTC in conjunction with a fluorometer and associated software was able to discriminate between background fluorescence in blood serum and the pyrene-tagged antibodies used in the immunoassay. It was also able to distinguish between multiple antigens associated with different decay times. There is a direct parallel, therefore, between the earlier DTC application and the identification of microorganisms. Both require the measurement of fluorescence decay times to an accuracy of 0.1 nanosecond, and both require the deconvolution and analysis of complex time decay functions.

Given the success of the earlier DTC development, what is required for the DTC as part of a laboratory prototype TRFF? The primary shortcoming of the earlier DTC was an uncertainty in the begin threshold signal at the peak of the excitation pulse. Laser pulse jitter and other signal variations caused a variation of the timing of the begin threshold signal that could be minimized only by long-time averaging of a number of probe measurements at each amplitude (and threshold) point on the decay time function. Development efforts will focus on the removal of this problem. Excessive time averaging is not desirable in a space station environment. Along with this major development thrust, however, there will be design changes to improve the overall performance of the DTC.

Decomposition Software

The LYNREG program, based on precise linear regression analysis, was briefly described in the previous progress report. Although LYNREG performed adequately in fluoroimmunoassay applications, an investigation of other methods with better real-time computation potential will be carried out. The Fast Fourier Transform (FFT) method with new developments in digital signal processing integrated circuits seems particularly promising. The FFT approach will be compared in speed and accuracy with the existing LYNREG program.

Pattern Recognition Software Development

The state-of-the-art in pattern recognition software, particularly with the rebirth of neural network methods, has advanced considerably in the past few years. The potential of neural nets and new versions of classical pattern recognition methods was discussed in the previous progress report. Since measurement noise is inevitable in an instrument such as TRFF, particularly at this 0.1 nanosecond measurement interval, it is important that the optimal techniques be used to finally identify the microorganism given the time wavelength-pattern vector. With an extensive library of pattern recognition software routines, OptoMed will be prepared to implement the best in pattern recognition techniques.

Conclusion and Recommendation

The "paper study" aspects of the TRFF version of an RTMM have reached their conclusion. Further, progress is possible only with the initiation of the proof of conception instrumentation (POCI) testing followed by the development of a Laboratory Prototype TRFF.

Kenneth J. Schlager

APPENDIX I

PROJECT TASKS
TRFF LABORATORY PROTOTYPE

1. Preliminary Measurements
   Use the POCI instrumentation and a high-speed oscilloscope to verify adequate photomultiplier (PMT) output and decay time functions of selected bacteria. This testing is intended to expose any basic problems in the design approach.

2. Master Schematic
   Prepare a master schematic of the TRFF.

3. Master Software Flow Chart
   Prepare a master flow chart of the TRFF software.

4. Interface Specifications
   Prepare interface specifications for the following interfaces:
   - CPU-Laser
   - CPU-Decay Time Computer
   - CPU-PMT Control
   - PMT-Decay Time Computer

5. Decay Time Computer Design
   a. Schematic
   b. ECL Layout
   c. Assembly Drawing
   d. Procurement of Parts

6. Interfaces Design
   a. Schematics
   b. PCB Layout
   c. Assembly Drawing
   d. Procurement of Parts

7. Operator Panel Design

8. Decay Time Computer Artwork Preparation

9. Interfaces Artwork Preparation

10. Software Development
    a. Decay Time Computer Control
    b. Laser Control
    c. PMT Control
    d. LYNREG Integration
    e. FFT Deconvolution
    f. Classical Pattern Recognition
    g. Neural Network Pattern Recognition

11. Assemble Decay Time Computer.
    Test and Modify DTC.

12. Assemble Interfaces.
    Test and Modify Interfaces.

13. Assemble TRFF System.
    Test and Modify TRFF.

14. Load and Test TRFF Software.

15. Test TRFF with microbiological samples.

Note: The above task summary assumes the existence of the Proof of Concept Instrumentation (POCI) hardware as a starting point for the project.

12825 Elmwood Road
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March 28, 1989

Mr. Melvin J. Kilgore
CONSORTIUM FOR THE SPACE LIFE SCIENCES
SPMC Clinical Science Center, CSC-213
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Huntsville, AL 35801

For consulting services rendered on the
Real-Time Microbiological Monitor (RTMM) Project $1,500.00
during the period ending March 31, 1989
Purchase Order Numbers: 003389, 003347

Kenneth J. Schlager, Ph.D., P.E.